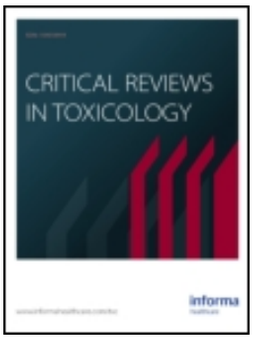

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REVIEW ARTICLE

Effects of atrazine in fish, amphibians, and reptiles: An analysis based on quantitative weight of evidence

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Abstract

A quantitative weight of evidence (WoE) approach was developed to evaluate studies used for regulatory purposes, as well as those in the open literature, that report the effects of the herbicide atrazine on fish, amphibians, and reptiles. The methodology for WoE analysis incorporated a detailed assessment of the relevance of the responses observed to apical endpoints directly related to survival, growth, development, and reproduction, as well as the strength and appropriateness of the experimental methods employed. Numerical scores were assigned for strength and relevance. The means of the scores for relevance and strength were then used to summarize and weigh the evidence for atrazine contributing to ecologically significant responses in the organisms of interest. The summary was presented graphically in a two-dimensional graph which showed the distributions of all the reports for a response. Over 1290 individual responses from studies in 31 species of fish, 32 amphibians, and 8 reptiles were evaluated. Overall, the WoE showed that atrazine might affect biomarker-type responses, such as expression of genes and/or associated proteins, concentrations of hormones, and biochemical processes (e.g. induction of detoxification responses), at concentrations sometimes found in the environment. However, these effects were not translated to adverse outcomes in terms of apical endpoints. The WoE approach provided a quantitative, transparent, reproducible, and robust framework that can be used to assist the decision-making process when assessing environmental chemicals. In addition, the process allowed easy identification of uncertainty and inconsistency in observations, and thus clearly identified areas where future investigations can be best directed.

Keywords

atrazine, amphibians, fish, reptiles, weight of evidence

History

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Abbreviations: *11-KT* 11-ketotestosterone, *17,20βP* 17,20β-dihydroxy-4-pregnen-3-one, *AChE* acetylcholine esterase, *ACTH* adrenocorticotropic hormone, *AOP* adverse outcome pathway, *APND* aminopyrine N-demethylase, *APVMA* Australian Pesticides &Veterinary Medicines Authority, *ar* androgen receptor, *ATV Abystoma tigrinum virus, Bd Batrachochytrium dendrobatidis, BrdU* bromodeoxyuridine, *CAT* catalase, *Chl-a* chlorophyll-a, *CYP19* aromatase, a member of the cytochrome P450 superfamily, *dbcAMP* dibutyryl cAMP, *DEA* de-ethyl atrazine, *EC50* concentration causing a stated effect in 50% of the tested individuals, *EO* early ontogeny, *EOG* electro-olfactogram, *er* estrogen receptor, *ERND* erythromycin N-demethylase, *FW* fresh water, *GLP* good laboratory practice, *GPx* glutathione peroxidase, *Gr* glucocorticoid receptor, *GR* glutathione reductase, *GSH* glutathione, *GSI* gonad-somatic index, *GST* glutathione-S-transferase, *hsp70* heat shock protein 70, *hsp90* heat shock protein 90, *iNOS* inducible nitric oxide synthase enzyme, *i.p.* intraperitoneal (injection), *IUCLID* International Uniform Chemical Information Database, K_{OC} partition coefficient between water and organic matter in soil, $K_{O/W}$ partition coefficient between octanol and water, *LO* late ontogeny, *LOEC* lowest observed effect concentration, *MDA* malondialdehyde, *MDA* Minnesota Department of Agriculture, *MoA* mechanism and/or mode of action, *MRC* mitochondria-rich cell, *mRNA* messenger RNA, *NAWQA* National Water-Quality Assessment, *NF* Nieuwkoop and Faber (stage of development of tadpoles), *NO* nitrous oxide, *OECD* Organization for Economic Cooperation and Development, *PGF2α* prostaglandin F2α, a pheromone released by female fish, *PSU* practical salinity units, *QA & QC* quality assurance and quality control, *ROS* reactive oxygen species, *SAP* Science Advisory Panel, *SCV* spring carp virus, *SD* standard deviation, *SE* standard error, *SEJ* score from expert judgment, *SI* supplemental information, *SOD* superoxide dismutase, *SOM* strength of method,

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SVL snout-vent length, *SW* salt water, *T* testosterone, *T3* triiodothyronine, *T4* tetraiodothyronine, *TBARS* thiobarbituric acid reactive substance, *TH* thyroid hormone, *TKN* Total Kjeldahl Nitrogen, the sum of organic and inorganic nitrogen, *TO* testicular oocytes, see TOF, *TOF* testicular ovarian follicle, *TWA* time weighted average, *USEPA* United States Environmental Protection Agency, *USGS* United States Geological Survey, *Vtg* vitellogenin, *WoE* weight of evidence, *WWTP* waste water treatment plant

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Introduction

The herbicide atrazine (CAS # 1912-24-9) has been under intensive scientific and regulatory review for over a decade due to its potential effects on growth, reproduction, and development in aquatic vertebrates, particularly amphibians. The scientific literature contains numerous studies linking responses to atrazine exposure, while many other studies report no evidence of response. The purpose of this analysis was to conduct a formal weight of evidence analysis on all available data related to atrazine and responses in fish, amphibians, and reptiles. A consistent, transparent, and defensible set of criteria were used to evaluate the strength of the studies with respect to the methods employed, ecologically relevant response endpoints (such as the apical endpoints of survival, growth, development, and reproduction) and environmentally realistic concentrations. The criteria developed allowed for straightforward scoring in two-dimensional plots and a means by which to assess the available evidence linking atrazine to ecologically-relevant responses in taxa of interest. Scoring was then conducted in an open and transparent manner.

The potential effects of atrazine on reproduction in mammals have been a subject of interest since the 1990s. The observation of an increased incidence of mammary tumors in Sprague Dawley rats led to several investigative studies in mammals that elucidated the mechanism of this strain-specific response. This led to the conclusion that this response was not relevant to humans (summarized in Solomon et al. 2008). Since 2008, a number of studies on the developmental toxicity of atrazine and its chloro- and hydroxyl- metabolites in mammals have provided more evidence to support the conclusion that, even at large doses that are toxic to the mothers (~75,000 to 100,000 μg/kg/day), atrazine is not a reproductive toxicant in mammals. No effects were observed on fetal viability or embryo development (Scialli et al. 2014), multigenerational reproductive performance (DeSesso et al. 2014, Foradori et al. 2014), and the hormonal processes involved in reproduction (Simpkins et al. 2011).

The lack of information on the potential effects of atrazine in development and reproduction in aquatic organisms was noted in the 1990s. A review and assessment of ecological risks in the aquatic environment from 1996 (Solomon et al. 1996) was conducted, with a primary focus on effects on fish, invertebrates, macrophytes, and algae, and measured environmental exposures. The topic was revisited and further refined in a book published in 2005 (Giddings et al. 2005). These assessments concluded that atrazine did not present significant acute or chronic risks to aquatic organisms at typical environmental concentrations found in N. America. However, both of these reviews noted that although there were robust data for effects in laboratory toxicity tests and meso- and micro-cosms that addressed responses in fish, invertebrates, and plants, there were few data on aquatic stages of amphibians and reptiles. In

addition, it was noted that effects on development and reproduction were not well-addressed in the literature available at that time.

The observations made in the late 1990s, of the effects of large doses of atrazine on reproduction in rats, and the lack of data on amphibians and reptiles, prompted the initiation of a number of laboratory and field studies in the early 2000s, with a focus on development and reproduction in amphibians and reptiles as endpoints. Some of these studies were conducted at the request of the registrant (Syngenta Crop Protection, LLC), and others by the research community at large. Most of these studies have been published in the peer-reviewed literature, starting in 2002, and have been the subject of review by the USEPA and numerous USEPA Scientific Advisory Panels (SAPs) (USEPA 2003, 2007a, b, 2012c) and other regulatory agencies (for example, APVMA 2008, 2010, MDA 2010a, b). A review of the studies published up to 2008 was conducted by an expert panel (Solomon et al. 2008). This was followed by several other reviews and analyses in journals (Hayes et al. 2011, Mann et al. 2009, Rohr and McCoy 2010a) and books (Bishop et al. 2010b). In addition, policy perspectives were published in a journal (Rohr and McCoy 2010b) and in an edited book (Hayes 2011).

Several approaches have been used to review the literature on this topic. In the panel-review (Solomon et al. 2008), guidelines for causality developed from those of Koch (1942), Bradford-Hill (Hill 1965), and IPCS (IPCS 2002) were used. These guidelines were based on temporality, strength of association, consistency, biological plausibility, and recovery. As explained in the conclusion of the panel-review, each of these guidelines was applied to data on a range of responses, from the biochemical to the population level, to assess the biological relevance of multiple lines of evidence (Solomon et al. 2008). They concluded that there was no strong evidence to suggest that atrazine caused adverse effects at environmentally relevant concentrations. An assessment of the sub-lethal effects of atrazine (and other agrichemicals) on anurans, and the potential for these substances to cause effects on populations in the field (Mann et al. 2009), came to a similar conclusion.

The effects of atrazine on anurans and reptiles were reviewed in a book-chapter by Bishop et al. (2010b). This review covered much of the literature included in the above reviews, but added additional material to ca. 2009. However, the review did not critically analyze the quality of the literature, nor did it the assess environmental relevance of the large exposure concentrations used in many laboratory studies. In addition, the review did not address the atypical concentration-responses reported by some authors. They pointed out that, for a number of endpoints, some studies show effects and others do not, and concluded that future studies should focus on the broader impacts of atrazine and its effects on habitat.

In the 2011 review, Hayes et al. (2011) used the Bradford-Hill guidelines to argue that atrazine has adverse effects on the gonads and/or their development across the vertebrate classes. However, the authors misapplied the Bradford-Hill guidelines by selecting observations that fulfilled the guidelines in one taxon, and then extrapolating these to all others. To support causality, the majority of the Bradford-Hill guidelines should be satisfied for each response in each species. In addition, they omitted literature that did not support their conclusion that atrazine was an endocrine disruptor. The omitted studies were arguably of demonstrably better quality, and the conclusions were supported by robust science. For example, there was no mention of the paper by Kloas et al. (2009a), which presents replicated studies that were requested by the USEPA, conducted under GLP with QA & QC, and independently assessed by a Science Advisory Panel (Grim and Steeger 2008, USEPA 2007a). Kloas et al. (2009a) showed that atrazine had no biologically significant effects on gonadal development or development to metamorphosis in *Xenopus laevis* when exposed to concentrations ranging from 0.01 to 100 µg/L. Moreover, other papers that identified intersex in frogs in urban and agricultural environments (Skelly et al. 2010), and the lack of effect of atrazine on gonadal development in frogs (Spolyarich et al. 2010), were also omitted. The review by Hayes et al. (2011) concluded that atrazine disrupts sexual development and reproduction by no less than nine different mechanisms that cut across taxonomic orders of animals that are widely different physiologically and biochemically. That a single chemical could act via all these different pathways—and at environmentally realistic exposures—would be most unusual; in fact, it would be astonishingly unique. The conclusions of Hayes et al. (2011) are also contradictory to those in reviews from independent regulatory agencies (APVMA 2008, 2010, MDA 2010a, b, USEPA 2007a, 2012c) and in the scientific literature (Mann et al. 2009).

Another approach to the topic, meta-analysis, was used in a review by Rohr and McCoy (2010b). This paper was a "qualitative meta-analysis" of information published up to 2009, and concluded that atrazine affected several endpoints related to development, reproduction and susceptibility to diseases. This analysis has several weaknesses. Firstly, a large number of papers and observations reported in studies were totally excluded from the analysis, many for reasons related solely to lack of detailed reporting of statistical analyses (author's supplemental information (SI) Table, which is unfortunately no longer available electronically). Secondly, a *qualitative* meta-analysis can be used to formulate hypotheses and theories but not make conclusions (Friedman 2000). In order to draw conclusions, a *quantitative* meta-analysis is required. A *quantitative* meta-analysis is a process or technique of synthesizing research results by using various statistical methods to retrieve, select, and combine results from separate but related studies. The major function of a meta-analysis is to overcome small sample sizes and increase precision of results of epidemiological and clinical studies (Friedman 2000). In their *qualitative* meta-analysis, Rohr and McCoy did not use the combined data from other studies in a quantitative manner and also did not address the influence of differences in 1) species, 2) magnitude of the effects, 3) exposure protocols, 4) experimental environments, and 5) endpoints. The method used for the *qualitative* meta-analysis was a simple vote-counting process and, as has been pointed out, this will invariably yield the incorrect conclusion if the power of the included studies is low (Friedman 2000). A more recent "meta-analysis" took a broader view of the effects of a range of chemicals on amphibians (Egea-Serrano et al. 2012). Although these authors included pesticides as one of the groups of chemicals, they did not specifically address the potential effects of atrazine and drew no conclusions.

Weight of evidence (WoE) has recently been identified as a useful and rigorous process for evaluating the results of scientific studies and combining these into an objective conclusion. In 2010, an editorial in Nature called for WoE approaches to assess studies for the purposes of regulatory decision-making (Nature 2010). The term "weight of evidence" is not new; it has been used for some time in the legal context (Krimsky 2005), as well as in the scientific literature. However, as has been pointed out (Weed 2005), the term is widely used in the literature and is most often used metaphorically, where WoE refers to a collection of studies or to an unspecified methodological approach, but the term and the associated process is seldom formalized. Other, less frequent uses of the term have been methodological, e.g., a systematic narrative review, a meta-analysis, causal criteria, and/or quality criteria for toxicological studies. The term has rarely been used as a method for quantitatively weighing evidence or as a conceptual framework, a conclusion also reached in recent reviews of the use of WoE in the literature (Exponent 2009, Linkov et al. 2009). More recently, WoE has begun to be used in more formal and quantitative ways in the assessment of risks related to carcinogenicity (Bailey et al. 2012, Rhomberg et al. 2010, Rhomberg et al. 2011), neurotoxicity (Prueitt et al. 2011), endocrine disruption (Borgert et al. 2011, Borgert et al. 2014, Rhomberg et al. 2012), and pesticides (Kier and Kirkland 2013). These papers have illustrated the advantages of a formal approach to WoE in terms of evaluating the quality of data and testing hypotheses of human-significance or causality. None of these papers tested hypotheses of causality in the formal statistical sense and exactly how this would be done has not been suggested.

We have developed and implemented a similarly formal and quantitative approach to a WoE analysis of the information pertaining to the effects of atrazine on fish, amphibians, and reptiles. We conducted the WoE analysis on 33 main responses from the cellular to the population level. For each of these responses, WoE was used to test the hypothesis that atrazine was the causative agent of the response. Where data were available, hypotheses for fish, amphibians, and reptiles were tested.

Methods

We developed a WoE process based on scoring for relevance of experimental results and strength of the methods (SOM) used in the study. In this sense, the method is similar to the quantitative scoring systems described in Weed (2005) and Linkov et al. (2011). Our WoE analysis was designed to minimize bias and to be transparent. The analysis included papers (and a few publicly available reports) on the effects of atrazine on fish, amphibians, or reptiles. We included studies published in the literature up to July 2014, and most were published after 2000. No studies were excluded based on predetermined quality attributes; however, some older reports from the gray literature were only available in summary form and it was not possible to include these in the WoE.

As the WoE approach assigned scores to the relevance of the observations and the strength of the study methods, we did not exclude studies that had one or more weaknesses; these were reflected in the scores for strength and relevance. This approach is different from that used in the USEPA's white paper prepared for the 2012 SAP on atrazine (USEPA 2011), where many studies were excluded on the basis of a quality assessment. Thus, we avoided the potential problem of selection bias (Walker et al. 2008). We conducted and continuously updated searches of the scientific literature through PubMed, SciFinder®, CAPLUS (via SciFinder®) Biosis Preview and CAB (via Ovid®), EMBASE, and Google® Scholar, in an attempt to capture all papers on the topic and minimize search bias. However, we were unable, for the most part, to avoid publication bias, i.e., the lower likelihood for papers reporting no adverse effects to be published (Walker et al. 2008). Thus, conclusions from the literature are typically biased towards the reporting of (adverse) effects. This bias is unquantifiable but was reduced to some extent by the inclusion of GLP studies required by regulatory agencies where all results, adverse or not, are "published". In general, publication bias is likely to make the conclusions of any WoE assessment more conservative.

Generally, most evaluations were on studies in which the exposure was to atrazine alone. Some studies on mixtures were included in the analysis of WoE. Although causality cannot generally be assigned to responses to a mixture of stressors where the individual components were not tested separately, studies on mixtures containing atrazine, where no effects were observed, were included in the analysis of WoE. A similar approach was used where formulated products, which contained other chemicals, were tested (see section on *Uses, chemical, physical, and biological properties*). Causality was not relevant in these studies as no effects were observed. Some papers were not subjected to WoE analysis but were still included in the narrative. Where papers provided information on responses that have been linked to atrazine but did not specifically report on experimental studies with this chemical, these were included for explanatory purposes.

Our WoE analysis was designed to provide the reader with a detailed assessment of each paper. The paper was initially assessed and scored by one of the authors, and then subjected to quality assurance by an outside expert who checked the accuracy of the descriptions of the methods and the cited data. After QA, the WoE assessment was reviewed by all authors for evaluation of clarity and consistency in scoring. This review was conducted face-to-face or in web-meetings. The analyses were grouped by type of response, each of which is graphically displayed and discussed separately. The supplemental information (SI) is in the form of a linked Adobe PDF file which allows access to the information used to develop the WoE analysis. Because many studies reported multiple responses, they were categorized under more than one section of the paper.

We developed criteria to characterize adverse outcome(s) in terms of biological relevance of the response and the SOM used to measure the response. This is similar to the WoE-method used to assess environmental impacts of an oil spill (McDonald et al. 2007). To assess these, we assigned scores to the criteria listed in Table 1. The criteria of relevance were based on "viewpoints" for causality from Hill and IPCS (Hill 1965, IPCS 2002), and are described in more detail below. We recognize that some of these scores, particularly the score for strength of the experimental design, were based on expert

Table 1. Attributes and scores for assessment of Weight of Evidence.

Score for relevance of response to adverse outcomes	0	1	2	3	4
Statistical significance for effects	Not statistically significant	Statistical significance inconsistent internally (e.g., in repeated observations within the study)			Statistically significant
Consistency and strength of concentration- or dose-response (C-R)	None, or $r^2 < 0.1$	$r^2 < 0.2$, evident but not characterized	$r^2 < 0.4$ or C-R with 2 concentrations only	$r^2 < 0.6$	$r^2 < 0.8$
Relevance of effect to survival, development, growth, and/or reproduction	None	Unexplained, biomarker response of low relevance or small non-critical response (< 5%)	Low (e.g., minor changes in weight or more relevant biomarker (< 10%))	Moderate (e.g., larger changes in weight or relevant biomarker (10%–25%))	High (e.g., any significant responses resulting in adverse effects at the level of the population.
Mechanism and or mode of action of adverse effect explained	No effect, highly implausible, or internally inconsistent	Not proposed or explained	Plausible but not characterized in study	Plausible based on analogous studies	Plausible and characterized in study
Multiplier for relevance of lowest observed effect concentration (LOEC)	Effects observed only at ≥ 100 μg atrazine/L = 0	Effects observed at < 100 μg atrazine/L = 1	NA	NA	NA
Overall evaluation of adverse outcomes relative to risk	Not relevant → Highly relevant Based on the computed mean of the individual scores and the concentration multiplier				
Overall evaluation of relevance score from expert judgment	A score for expert judgment (SEJ) where exceptions such as internal inconsistency, incorrect statistical analysis, or husbandry issues, etc. were identified and a score greater or less than the computed score assigned.				
Strength of methods (SOM)	**0**	**1**	**2**	**3**	**4**
Experimental design and hypotheses	Completely inappropriate	Major weakness(es)	Some weakness(es)	Good	Excellent
Use of GLP and QA/QC	None	Some QC, on regular basis (e.g., some measured exposures)	More QC (e.g., exposures measured on a regular basis)	Spirit of GLP with QA/QC	GLP with QA/QC
Transparency of data	Critical data not provided	Summary and aggregate data provided			All raw data available for review
Concentrations of atrazine tested	1	2	3 or field study ≥ 3 sites	4	≥ 5
Use of realistic atrazine concentrations	All > 100 μg/L	All > 20 μg/L	1 < 20 μg/L	2 < 20 μg/L	≥ 3 values < 20 μg/L or a field study
Overall evaluation of the strength of the methods	Weak methods→ → → → → → → → → → → → → → → →→ → → → → →→ → → → → →→ → → → →→ → Strong methods Based on the computed mean of the individual scores				

judgment and are subject to potential bias. However, the weaknesses in the design of the study are clearly identified in the WoE (see SI) and are fully transparent. Other scores were based on numerical values from the study and are without bias. In all, over 1290 responses were scored.

Criteria for scoring relevance of the response to adverse outcomes

Details of the scoring of relevance (Table 1) are described below:

- The scoring criterion for statistical significance of effect was based on significance at a P-value less than 0.05. Whether the outcome was adverse or not, a score of 4 was assigned. Outcomes identified as non-adverse (e.g., increased survivorship) were addressed in the score from expert judgment (SEJ). If only some of the repeated trials in the study were statistically significant, scores were between 1 and 3.
- Concentration- or dose-responses were scored as indicated (Table 1). For kinetic reasons, the most commonly observed response of an organism to exposure to a substance is monotonic. Non-monotonic dose- or concentration- responses have been reported for some chemicals (Vandenberg et al. 2012). Non-monotonicity is usually observed for receptor-based interactions of hormones and their agonists; typically, these are observed at greater concentrations *in-vitro,* and are rarely seen in apical endpoints after small and/or long-term exposure (USEPA 2013). Non-monotonic responses are biologically plausible, and as pointed out (USEPA 2013), can result when the response of the biological system that is the target of the chemical consists of two or more activities that might act in opposition to each other. There is no mechanistic evidence to suggest that atrazine acts in this way in target or non-target organisms (Solomon et al. 2008). It is not an agonist or antagonist of any of the steroid hormones, and interaction with aromatase has been shown to be monotonic in *in-vitro* studies (Sanderson et al. 2000, Sanderson et al. 2001). Therefore, non-monotonic responses were assigned a score of zero unless a plausible mechanism, such as those described in (USEPA 2013) was demonstrated, and there were more than two concentrations on either side of the inflection to properly characterize the response as non-monotonic. If necessary, this was addressed in the narrative and the SEJ. Thus, a statistically significant response only at a low or intermediate concentration received a smaller SEJ but non-significant responses that demonstrated a consistent concentration-response would receive a greater score, as this may be indicative of a causal relationship. If a coefficient of correlation (i.e., r^2) was calculated, this was also used as a criterion for scoring.
- Relevance of adverse effects to the apical endpoints of survival, development, growth and/or reproduction was scored as indicated (Table 1). The use of these apical endpoints is common in ecological risk assessment, where the emphasis is on populations rather than individuals. Where no statistically significant effect was observed, the score was zero. Where statistically significant responses were observed, scores (1–4) were assigned in relation to the nature of the response, using the concepts from adverse outcome pathways (AOPs, Ankley et al. 2010) and biological cascades (Brain and Brooks 2012). For example, a small change in a biomarker response of low relevance received a lower score than a larger change in a relevant biomarker. A score of 4 was assigned to any statistically significant responses resulting in clear adverse effects at the level of the population. Positive responses, such as increased weight or size, were not considered adverse and received a score of zero, unless this was linked to other negative outcomes.
- The scoring of mechanism and/or mode of action (MoA) of the adverse effect is an assessment of the plausibility that the observed response is causally linked to atrazine. A score of zero was assigned if there was no effect, or if the MoA was highly implausible or internally inconsistent with other observations in the paper. Other scores ranged from "not proposed or explained" (score = 1) to "plausible and characterized in the study" (score = 4).
- The multiplier for relevance of concentration was based on a lowest observed effect concentration (LOEC) of atrazine at 100 μg/L or greater. As it was not possible to easily integrate concentration-response into the WoE diagrams, this was considered in the WoE by using a concentration-multiplier based on the lowest concentration of atrazine at which adverse effects were observed. As atrazine concentrations of 100 μg/L or greater are very infrequently observed (or estimated) in surface waters (see section on *Concentrations of atrazine in the environment*), this was used as a threshold value for assessing the relevance of responses only observed at large concentrations in the laboratory or field. If the LOEC was 100 μg atrazine/L or greater, the multiplier was zero; if the LOEC was less than 100, the multiplier was 1. In the cases when no effects were observed (score for statistical significance is zero), a multiplier of 1 was used to allow a score for reported or observed concentration-response to be included. This approach was taken because this paper is focused on determining the evidence for effects of atrazine under real world conditions, hence the importance of both the exposure assessment and the consideration of only effects, at these environmentally relevant concentrations
- The overall evaluation of adverse outcomes relative to risk was derived in two ways. The computed score was the mean of the other scores, and ranged from 0 to 4. This was then multiplied by the score for relevance of concentration. Another score (the score for expert judgment (SEJ)) was sometimes assigned when the computed score was determined by expert judgment (i.e., the authors) to be of lower relevance to adverse effects. As plotting the original score and the score modified by SEJ on the graphs obscured other data, the SEJ values are identified in the graphic display with a different symbol, explained in the WoE analyses (see SI), and addressed in the narrative. Of the total number of responses subjected to WoE analysis (1,290), approximately 14% of the computed scores were reduced after expert review. Less than half of the reduced SEJ values (6% of all responses) were because of inconsistency of the response, a lack of concentration-response, or a failure to demonstrate possible causality. A similar number of SEJ values were reduced for a variety of reasons that related to design and conduct of the study, evidence of poor husbandry, or statistical weaknesses that impaired the ability to

ascertain the relevance of the response. For example, some studies inappropriately reported reproductive endpoints or behavioral observations in animals experiencing mortality or severe sublethal effects. In a relatively small number of cases (~2% of all responses), a reduced SEJ value was assigned because the response was not considered adverse, such as a change in the activity of liver enzymes related to the metabolism and elimination of xenobiotics.

For all of the above criteria, if the author(s) of the study under review had made an error in analysis and the raw data were available, these were reassessed and the score adjusted upwards or downwards as appropriate. In other cases, scores for concentration-response in the absence of statistical significance were increased, even if the relationship was not characterized but was obvious from the data provided.

Strength of the methods used in the study

Scoring for the methods of the study and the procedures (Table 1) was similar to that recommended for assessing studies for inclusion in the International Uniform Chemical Information Database (IUCLID, Klimisch et al. 1997) and for setting criteria for environmental quality (Breton et al. 2009). Scoring approaches were also similar to those recommended by the USEPA for assessing studies from the open literature (USEPA 2011, 2012a). The scores are described below.

- The experimental design and appropriateness of the hypotheses were scored from 0 to 4 (Table 1). A score of 0 was assigned if the design was deemed completely inappropriate and a score of 4 assigned if it was considered excellent. A summary of the methods used, weaknesses identified, and a rationale for the score, are provided in the analysis of each WoE (see SI). If no concerns were identified, the score was 4, but where one or more weaknesses were identified, the score was reduced accordingly. Examples of common weaknesses included: insufficient description of the methods to allow a full evaluation, inappropriate experimental design, lack of information about numbers of treatments, replicates, or test subjects per replicate, lack of description of the purity or form of the test substance, inappropriate statistical comparisons, lack of appropriate controls, lack of details about husbandry of organisms, evidence of poor husbandry, etc. Weaknesses were identified as major if they were serious omissions or design faults that may have compromised the results and conclusions of the study, or minor if they were less serious (e.g., the provision of insufficient detail of the methods).
- The use of GLP and QA & QC was scored from 0 to 4 (Table 1). Studies required by regulatory agencies are usually carried out according to accepted guidelines and under GLP with QA & QC. As has been pointed out, peer review is not a reliable process for establishing the quality of data (McCarty et al. 2012), mainly because the raw data are usually not provided to the reviewer. In a GLP study, all the raw data are available for review and/or reanalysis, and all results are provided, thus avoiding publication bias. Increased availability of additional data through supplemental information (SI) is a step towards greater transparency (McCarty et al. 2012), but these data are not the equivalent of those supplied in a GLP study. While a GLP study may not always address the correct question (which is scored separately in this WoE in Experimental Design and Hypotheses), the methods are transparent and, if published and subjected to peer-review, bring the best of both worlds to the process by making reliable and appropriate data freely available. If there was no GLP or QA/QC, the score was 0. Full GLP with full QA & QC was assigned a score of 4. The spirit of GLP with QA & QC was defined as a study with a specific protocol, a record of deviations from the protocol, and a full set of raw data available for review, but without QA. This was assigned a score of 3. Scores between 0 and 3 were assigned based on the amount of QC, such as, for example, measurements of actual exposure concentrations at the start of the study only (score = 1), or measurements of exposures and other parameters (e.g., those related to water quality, such as dissolved oxygen) at regular intervals (score = 2).
- Transparency of data was scored from 0 to 4 (Table 1). If critical data were not provided, a score of 0 was assigned. If full raw data were available, the score was 4. If data were provided in tables and graphs, intermediate scores were assigned depending on the clarity of the data and the description of variance, etc. For example, if data were in graphs and tables and measures of variance (standard error (SE) or standard deviation (SD)) were provided, a score of 2 was assigned.
- Since response to concentration is a key determinant of causality and a basic principle of toxicology, the greater the number of concentrations tested, the easier it is to characterize the concentration- or dose-response and calculate margins of safety. If only one concentration was used, a score of 0 was assigned, with greater scores for more concentrations tested. The maximum score of 4 was assigned to studies that utilized 5 or more concentrations, the number recommended for many guideline studies such as those of the OECD or USEPA. If the study was a field study conducted with samples from 3 or more sites, a maximum score of 2 was assigned due to the lack of control over actual exposures between sites and over the time and suitability of the reference sites.
- The use of environmentally realistic exposures was scored from 0 to 4 (Table 1). As the upper centiles of measured and estimated exposures of atrazine in surface waters are ca. 20 µg/L (see discussion below), this was defined as a realistic exposure. This score is related to the concentration-multiplier (above) for relevance, but here, it applies to the need for studies to include some environmentally relevant concentrations if they are to be useful in extrapolations of risks and assessing the relevance of environmental exposures. If all concentrations were unrealistic (defined here as greater than 100 µg/L), a score of 0 was assigned. If the concentrations tested included one value of 100 µg/L or lower, the score was 1, if one value was 20 µg/L or lower, the score was 2, if there were two values of 20 µg/L or lower, the score was 3, and if three or more values were 20 µg/L or lower, or it was a field study, a score of 4 was assigned. Durations of exposure were not considered in this assessment, due to the difficulty in categorizing the myriad of concentration/duration combinations but, where

appropriate, were considered in the SEJ, and are discussed in the narrative.
- The overall evaluation of the SOM was determined from the computed average of the scores for the above criteria.

The mean scores for the relevance and SOM for each study have no meaning other than for the purposes of ranking. To visualize these scores, they were plotted on the X- and Y-axes of a graph (Figure 1), thus allowing all of the studies that reported a particular response to be visually displayed in a summary evaluation. The location of the point within a quadrant provides a measure of the strength of the methods and the relevance of the findings to adverse effects for that specific study and response. Obvious outliers were then easily identified and were addressed in the narrative. The absolute scores have no real meaning; it is the relative scores that are important. The purpose of the scores is to separate the more relevant from the less relevant and the strong from the weak. Clustering of the studies in a particular part of the graphic is indicative of consistency between observations in different studies. For each response assessed, the mean and 2 × SEs of the scores for strength and relevance across all WoE assessments were calculated and plotted on the graphs as a summary of the individual response (Figure 1). The same plots are presented in the SI, where they are linked to the detailed individual WoE analyses from which the scores were obtained, and where the evaluations of the studies are provided.

As discussed above, hypotheses of causality were not tested in the statistical sense, but the mean scores for SOM used in the studies (and their uncertainty) were used to gauge the overall quality of the evidence. The mean scores for relevance of the results to apical endpoints (and their uncertainty) provided a simple indicator for the hypothesis of causality, according to the following scheme:

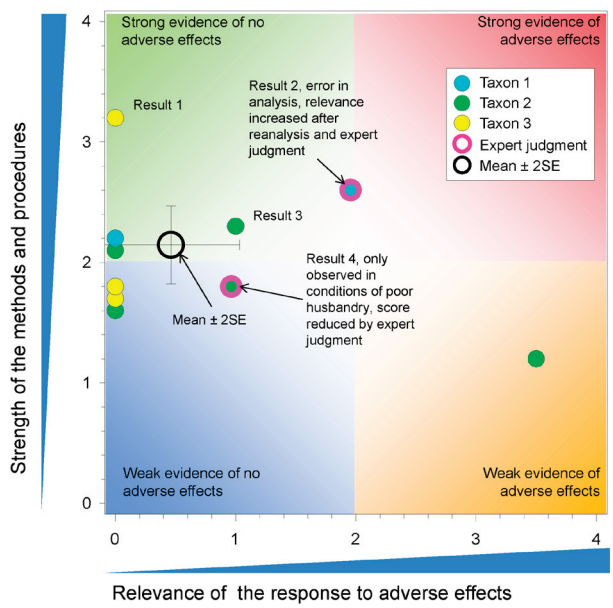

Figure 1. Illustration of the graphical presentation of the WoE for a particular response. Results, each a separate observation in a paper or report, are represented by the colored circles. These are identified and linked to the WoE in the SI. The mean and 2 × SE of the scores are shown by the symbol with the error bars.

If the mean score for relevance was zero, the null hypothesis of causality (that atrazine, at concentrations commonly found in the environment, does not cause effect "x") was clearly considered not falsified. When the score for relevance was 3 or greater, the null hypothesis was considered falsified. For example, the relevance for the effects of estradiol on sexual determination (see Figure 4 in Solomon et al. 2008) would achieve scores of 4, from 1– 4, 4, and 4 for statistical significance, concentration response, relevance, and mechanism (Table 1), respectively, for an average of 3.25 – 4.0 (without the LOEC multiplier). If the mean score was greater than 1 or less than 3, we considered that there was equivocal evidence that the null hypothesis was not falsified. A further qualifier related to uncertainty (SE of the mean score) was noted where this was equal to or greater than the mean score.

Uses, chemical, physical, and biological properties of atrazine

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine; CAS #1912-24-9) was introduced as a herbicide in 1957 (BCPC 2003), patented in Switzerland in 1958 and registered for commercial use in the United States in 1959 (Giddings et al. 2005). Atrazine is widely used for the control of certain annual broadleaf and grass weeds in corn, sorghum, sugarcane, and other crops. The principal registrant of atrazine is Syngenta Crop Protection, LLC, but it is manufactured and registered under license to several other agrochemical companies. Atrazine was widely used in the above-mentioned crops in the 1970s, but since that time, its use has declined significantly with the introduction of genetically modified crops that are resistant to other herbicides. Globally, at this time, it is among the most widely used herbicides in corn, with benefits of increased yields and decreased erosion and siltation of surface waters through compatibility with no-till agriculture. Between 2000 and 2010, annual use of atrazine in North America was about 33 million kg of atrazine on about 29 million ha of farmland (USEPA 2012b). In the U.S., rates of application of atrazine (2006–2010) for corn, sorghum and sugarcane were 1.12, 1.12, and 2.58 kg/ha, respectively. On average, about 20% - 40% of these crops are treated twice in one year (USEPA 2012b).

The environmental behavior of atrazine, its use patterns, mechanisms of action, and its bioconcentration/bioaccumulation, have been discussed in detail in previous reviews (Giddings et al. 2005, Solomon et al. 1996, Solomon et al. 2008) and are not repeated here in detail. Atrazine has a small K_{OC} (40 to 394) and relatively large solubility in water of 33 mg/L, which, combined with its persistence in soils and water of pH > 6.5 (Giddings et al. 2005), means that it can be mobile in soil and might be found in surface-and ground-water in areas where it is used in agriculture Atrazine does not bioaccumulate in organisms and does not biomagnify via the food chain, because of its low log $K_{O/W}$ (2.56 – 2.61) and rapid metabolism in animals.

The primary mechanism of action of atrazine as an herbicide is through the reversible inhibition of photosynthesis in photosystem II. This mechanism is specific to plants and has been discussed before (Giddings et al. 2005, Solomon et al. 1996). Other possible mechanisms of action related to adverse effects in organisms other than plants, both direct and indirect, are discussed below in the context of the analysis of WoE.

In the environment, atrazine is applied as a formulated product. The formulated product contains atrazine as the active ingredient, along with other ingredients. These other ingredients may be other herbicides (pre-mixed formulation) and formulants (so-called inert ingredients). The role of these formulants is to facilitate the application of atrazine in the field; some may be surfactants and/or agents to stabilize slurry formulations. These ingredients are not listed on the label of the product, but are known to, and have been reviewed by regulatory agencies such as the USEPA. To perform their function, these substances have properties that are different from those of atrazine, and once in the environment, their fate is not the same as that of atrazine. If atrazine were sprayed directly over water, the toxicity of these products and how they interact with atrazine in non-target organisms would be relevant to an assessment of risks to aquatic organisms such as fish, amphibians, and reptiles. However, atrazine is not labeled for over-water use, and when used on land, the formulants move and dissipate in ways different from those of atrazine. This means that concentrations of the active ingredient, atrazine, measured in matrices such as surface water, cannot be related to concentrations of formulants. For this reason, the use of formulated products in toxicity testing is inappropriate for risk assessment, unless the organisms in question or their habitat are sprayed directly. A number of studies have reported the effects of formulations of atrazine on aquatic organisms, but these cannot easily be related to concentrations in the environment and cannot be used to assign causality. These studies were assessed in the WoE. If they showed no response, it was concluded that the mixture of atrazine and the formulants had no effect at the concentration of atrazine tested and this was used in the assessment. However, if there were some effects, causality was not clearly attributable and this was noted with a qualifier in the text.

Concentrations of atrazine in the environment

Concentrations of atrazine in the aquatic environment have been measured and estimated using models. These have been discussed in reviews (Giddings et al. 2005, Solomon et al. 1996) and in more recent papers (Andrus et al. 2013, Battaglin et al. 2009, Brun et al. 2008, Kurt-Karakus et al. 2011, Woudneh et al. 2009a, b). Additional data are continuously added to the NAWQA database (USGS 2013). Based on previous reviews, more than 95% of the 90th centile concentrations of atrazine measured in surface waters across the US were less than 20 μg/L (Giddings et al. 2005). Refined Tier-4 modeling for concentrations in flowing waters and ponds provided annual maxima that were less than 10 μg/L. The data on concentrations measured in lotic waters have been reviewed in detail (references above), but we have highlighted some recent studies on lotic and lentic systems and also discussed specific issues related to environmentally relevant concentrations.

Measurements of concentrations of atrazine in ponds have been made less frequently, but several studies, particularly those conducted between 1970 and 1990 in agricultural settings, reported high concentrations (>300 μg/L). Some of these early studies have been used to justify high "environmentally realistic concentrations" of atrazine for the purposes of risk assessment for amphibians (Rohr and McCoy 2010b); however, these are not representative, even of reasonably worst-case scenarios. In some cases, published data have been misinterpreted. For example, the "pools" sampled by Edwards et al. (1997) were small-volume, water-filled, ephemeral depressions with very short persistence (<1 h) that are not a suitable habitat for aquatic organisms. The experimental study in Iowa by Baker and Laflen (1979) measured concentrations in wheel tracks, runoff, and soils, and not in pools, and the time-scale was less than a hundred minutes, so again, the system was not representative of the habitat of aquatic organisms. In addition, the simulated rainfall event used in the study was a one in a hundred-year event and therefore, is not typical. The study by Kadoum and Mock (1978) was based on measurements in irrigated fields in Kansas, where storage areas for tail-water were sampled. Median concentrations from over two years of collection and analysis of water samples (308 in total) ranged from 3.5 to 8.6 μg/L, but a maximum concentration of 1074 μg/L was observed on one occasion, and so again, is not representative of the current uses of atrazine.

More importantly, these older data on exposure concentrations also are not relevant to current use-practices for atrazine. The rate and frequency of the application of atrazine have decreased significantly since the 1970s, and the environmental concentrations measured have been observed to decline. A study by Kolpin et al. (1997) reported decreases between 1987–1991 and 1992–1995, that were consistent with reductions in rates of application over those periods. More recent measurements in ponds, pools, and ditches revealed that, although atrazine was above the level of detection (LOD) in 59% of the samples, the maximum concentration measured was 26 μg/L (Battaglin et al. 2009). A study on temporal trends and exposures in an area of intensive use of atrazine in South Africa showed concentrations less than 9 μg/L in a large number of samples (208) taken over an entire growing-season (Du Preez et al. 2005b). Measurements of concentrations of atrazine in lakes in Ontario, Canada, revealed that while 82% of samples had concentrations greater than the LOD (3×10^{-7} μg/L), the median value was only 0.002 and the maximum was 0.037 μg/L (Kurt-Karakus et al. 2011). This same study reported that 88% of samples of precipitation in Ontario had concentrations greater than the LOD (6×10^{-7} μg/L), with a median value of 0.0037 and a maximum of 0.043 μg/L, suggesting that there may be some transport via the atmosphere, possibly associated with particulates, as volatilization is unlikely to account for this based on the vapor pressure of 3.85×10^{-2} mPa (BCPC 2003). A study in British Columbia, Canada, reported maximum concentrations of atrazine in surface waters of 0.053 μg/L in agricultural sites and 0.008 μg/L in urban sites (Woudneh et al. 2009b). A field study conducted by the registrants of atrazine, as a requirement of the USEPA, measured concentrations of atrazine in surface waters of small watersheds in the U.S. that were assessed to be representative of those most vulnerable to runoff and represent an extreme worst case. Samples collected every 3 hours were composited and measured daily between 2010 (36 sites) and 2012 (31 sites, Table 2) during the atrazine-use season when runoff would occur. The measured values showed that less than 3% of the events exceeded 20 μg/L (rolling average concentration) for 4 days (acute exposures) and that less than 1% exceeded this concentration for 60 days (chronic). Concentrations greater than 100 μg/L were observed less than one percent of the time

Table 2. Percent of exposure events where the rolling average concentration for 4, 14, 30 and 60 d exceeded 10, 20 and 100 µg atrazine/L at streams in the U.S. in areas of high vulnerability to runoff[a].

Rolling average concentration of atrazine (µg/L)	Percent of sites where the rolling average exceeded 10, 20, or 100 µg atrazine/L for more than 4, 14, 30, or 60 d			
	4-d	14-d	30-d	60-d
> 10	5.86	6.19	5.43	4.19
> 20	2.31	1.75	0.97	0.50
> 100	0.06	0.03	0.00	0.00

[a]http://www.epa.gov/pesticides/reregistration/atrazine/atrazine_update.htm#ewmp.

and were all less than 14 days in duration. Thus, the claim that concentrations near 500 µg/L are ecologically relevant (see below) does not reflect observations from current environmental monitoring studies, even in regions with extensive atrazine use and/or extreme vulnerability to runoff.

Rather than use the large database on measured concentrations of atrazine, Rohr and co-authors (2008a, 2010b, 2011) used a Tier-1 model, GENEEC2 (USEPA 2001), to estimate environmental concentrations of 102 µg atrazine/L in ponds (Rohr et al. 2008a), and then argued, on the basis of historical (1960s) rates of application, that 500 µg/L is an "ecologically relevant concentration". The GENEEC2 model is a conservative Tier-1 exposure model used as a screen and designed to "predict values higher than most of the upper level concentration values that are measured at vulnerable sites in the field". More refined and appropriate models have been developed which predict concentrations of atrazine in ponds, from 0.001 to 30 µg/L, with a 95th centile concentration of less than 7 µg/L (Giddings et al. 2005); values that are consistent with those measured in surface waters in studies during the past 20 years. In light of the above discussion, we used a concentration of 20 µg/L as a criterion for design of studies in the laboratory and the field (Table 1). Based on the very small probability of the occurrence of concentrations greater than 100 µg atrazine/L in lotic and lentic waters, we used this criterion for the concentration multiplier (Table 1) for characterizing risks and as a threshold for classifying experimental exposures as unrealistic. Where effects were only observed at concentrations greater than 100 µg atrazine/L, we note this in the text with a qualifier "(> 100 µg/L)".

Since atrazine is not bioaccumulated or biomagnified (Giddings et al. 2005), exposure concentrations via diet were assessed for relevance using a default BCF of 1 (µg/L = µg/kg). For the same reason, the duration of exposure was not considered in the scoring, although it is reported in all WoE analyses. However, the durations of exposure in many of the tests, particularly those assessing development of amphibians, were fifty days or more. These longer studies are conservative for assessing risks in flowing waters and more representative of ponds, although concentrations in ponds do fluctuate as well (Du Preez et al. 2005b). Unless the experiment was specifically conducted at varying concentrations, the concentration was considered to be constant over the exposure time.

Effects of atrazine in aquatic organisms

Acute lethality and some chronic effects of atrazine on aquatic animals and plants were reviewed previously (Giddings et al. 2005). The effects of atrazine in algae and aquatic macrophytes are mediated by the well-known reversible effect of triazine herbicides on photosynthesis and are not discussed in detail here, except in the context of effects on primary producers that could lead to indirect effects on other organisms. Since early 2000, a number of reports of sublethal effects from acute and chronic exposures to atrazine in laboratory and field studies have been published in the literature. These are addressed in the following sections and are the primary subject of the WoE analysis in this paper. These effects are grouped by category (see SI) and the list of species and common names for all fish (n = 31), amphibian (n = 31), and reptile (n = 8) species included in this WoE are provided in Table 3.

In keeping with current taxonomy, we have used the current official name of the species, e.g., the genus *Lithobates* for *Rana,* in the discussion on the outcomes of the WoE analyses. However, we have not changed the name of the genus in the bibliography or in the individual WoE analyses, in order to maintain consistency with the original reports.

In the following sections, we have divided up the responses into several groupings for ease of discussion. Some of these relate to apical endpoints and others to responses, such as biochemical and physiological responses, which might be on the adverse outcome pathway to apical endpoints.

Direct effects of atrazine on development

All of these responses are directly related to development in that the effects might affect the successful maturation of the animals and thus the sustainability of the population over time. These responses span the entire life cycle of the organism.

Hatching of eggs in fish, amphibians, and reptiles

Even in R-strategists with high fecundity such as fish, amphibians, and many reptiles, hatching is an apical endpoint in terms of survival. Here, the null hypothesis tested by WoE was that atrazine, at concentrations commonly found in the environment, has no effect on hatching of eggs. Several studies have reported the effects of atrazine on this process, and the data for 26 responses are summarized in Figure 2 and discussed below. These studies were of varying duration but usually spanned the entire pre-hatch period (see SI).

Fish

Two laboratory studies were carried out with the fish *Pimephales promelas* (Bringolf et al. 2004, USEPA 2005) and showed no effects on hatching at concentrations of atrazine ranging from 5 to 250 µg/L (Figure 2). In a full life-cycle study, no effects on F_0 and F_1 hatching success of *P. promelas* were observed at exposures to atrazine at concentrations ranging from 150 to 2000 µg/L (Dionne 1992). In other full life-cycle studies, no effects on percentage of F1 egg hatching were reported in *P. promelas* (43 weeks, 15–213 µg atrazine/L parental exposure), *Lepomis macrochirus* (18 months, 8–95 µg atrazine/L parental exposure) or *Salvelinus fontinalis* (306 days, 65–720 µg atrazine/L parental exposure) (Macek et al. 1976). A study on eggs of *Oryzias latipes* (Papoulias et al. 2014) exposed to atrazine at concentrations ranging from 0.5 to 50 µg/L for 14 and 38 days showed no differences in

Table 3. Scientific and common names of fish, amphibians, and reptiles included in the analysis of WoE.

Scientific name	Common name
Fish	
Caquetaia kraussii	Basketmouth cichlid
Carassius auratus	Goldfish
Channa punctatus	Spotted snakehead
Chrysichthyes auratus	Golden Nile catfish
Colossoma macropomum	Tambaqui
Ctenopharyngodon idellus	Grass carp
Cyprinus carpio	Common carp
Danio rerio	Zebrafish
Fundulus heteroclitus	Mummichog
Gasterosteus aculeatus	Threespine stickleback
Gobiacypris rarus	Chinese rare minnow
Ictalurus punctatus	Channel catfish
Lates calcarifer	Barramundi
Lepomis macrochirus	Bluegill
Melanotenia fluviatillis	Australian rainbowfish
Micropogonias undulatus	Atlantic croaker
Micropterus dolomieu	Smallmouth bass
Micropterus salmoides	Largemouth bass
Oncorhynchus kisutch	Coho salmon
Oncorhynchus mykiss	Rainbow trout
Oncorhynchus tshawytscha	Chinook salmon
Oreochromis niloticus	Nile tilapia
Oryzias latipes	Japanese medaka
Pimephales promelas	Fathead minnow
Poecilia reticulata	Guppy
Prochilodus lineatus	Streaked prochilod
Rhamdia quelen	South American catfish
Salmo salar	Atlantic salmon
Salvelinus fontinalis	Brook trout
Sarotherodon mossambicus [Oreochromis mossambicus, Tilapia mossambica]	Mozambique tilapia
Sciaenops ocellatus	Red drum
Amphibians	
Acris crepitans	Northern cricket frog
Ambystoma barbori	Stream-side salamander
Ambystoma maculatum	Spotted salamander
Ambystoma tigrinum	Tiger salamander
Ambystoma texanum	Small-mouthed salamander
*Bombina pachypus**	Yellow-bellied toad
Bufo americanus	American toad
Bufo boreas	Western toad
Fejervarya limnocharis	Rice frog
Hyla versicolor	Gray tree frog
Limnodynastes fletcheri	Barking marsh frog
Limnodynastes peronii	Striped marsh frog
Limnodynastes raniformis	Growling grass frog
Limnodynastes tasmaniensis	Spotted grass frog
*Lithobates blairi**	Plains leopard frog
Lithobates catesbeianus [Rana catesbeiana]	American bullfrog
Lithobates [Rana] clamitans	Green frog
Lithobates [Rana] palustris	Pickerel frog
Lithobates [Rana] pipiens	Northern leopard frog
Lithobates [Rana] sphenochephala	Southern leopard frog
Lithobates [Rana] sylvatica	Wood frog
Osteopilus septentrionalis	Cuban treefrog
Pseudacris crucifer	Spring peeper
Pseudacris regilla	Northern Pacific tree frog
Pseudacris triseriata	Western chorus frog
*Rana aurora draytonii**	California red-legged frog
Rana luteiventris	Columbia spotted frog
*Rana mucosa**	Mountain yellow-legged frog
Rhinella arenarum	Common South American toad
Rhinella marina	Cane toad
Spea intermontana	Great Basin spadefoot
Xenopus laevis	African clawed frog
Reptiles	
Alligator mississippiensis	American alligator
Caiman latirostris	Caiman
Chelydra serpentina	Common snapping turtle
Graptemys ouachitensis	Ouachita map turtle
Graptemys pseudogeographica	False map turtle
Nerodia sipedon	Northern water snake
Thamnophis m. marcianus	Marcy's checkered garter snake
Trachemys [Pseudemys] s. elegans	Red-eared slider

*These amphibians not specifically tested with atrazine.

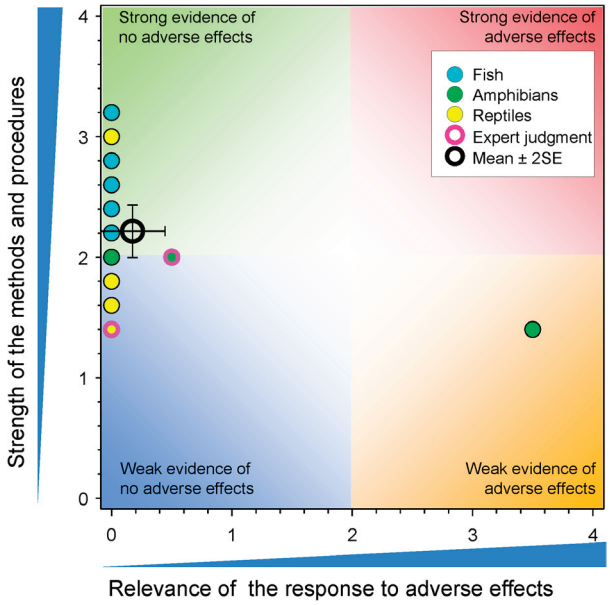

Figure 2. WoE analysis of the effects of atrazine on hatching in fish, amphibians, and reptiles.

the survival of eggs but mortality was large in all treatments, suggesting problems with husbandry.

Amphibians

Several studies were conducted in six species of frogs (Allran and Karasov 2001, Bishop et al. 2010a, Du Preez et al. 2008) and two species of salamander (Olivier and Moon 2010, Rohr et al. 2003, Rohr et al. 2004). The hatchability of eggs of *Lithobates [Rana] pipiens, Lithobates [Rana] sylvatica*, or *Bufo americanus,* from egg collection to 6 days, was unaffected at concentrations of up to 20,000 µg atrazine/L under laboratory conditions (Allran and Karasov 2001). No effects on hatching were reported in *X. laevis* at concentrations ranging from 1 to 25 µg atrazine/L (Du Preez et al. 2008). The hatching success of caged eggs of *Spea intermontana*, *Pseudacris regilla*, *Bufo boreas,* and *Rana luteiventris,* in response to pesticide exposure and varying parameters of water-quality, was studied in the field (Bishop et al. 2010a). Correlational analysis suggested a link between the presence of atrazine and hatching success in *S. intermontana* and *P. regilla* (but not *B. boreas,* and *R. luteiventris*). However, there were many confounding factors that might have affected hatching, and because it was not possible to assign causality of this response to atrazine, the SEJ was reduced. The exposure concentrations measured in these ponds were 0.08 µg atrazine/L or lower (Bishop et al. 2010a). It is considered unlikely that the eggs of these species would be more sensitive to atrazine by over 5 orders of magnitude than the species tested by others (e.g., Allran and Karasov 2001), although laboratory studies would be required to confirm this.

The two studies on the salamander *Ambystoma barbouri* were inconsistent; one reported no effects on hatching at atrazine concentrations up to 400 µg atrazine/L (Rohr et al. 2003), while the other reported effects on hatching at an atrazine concentration of 400 µg/L (Rohr et al. 2004).

A study on the effects of atrazine on the hatching of eggs of *Ambystoma maculatum* showed significant reduction in hatching at concentrations of formulated atrazine ranging from 50 to 400 µg/L (Olivier and Moon 2010). This species is biologically unique in that it is one of the few amphibians known to harbor an endosymbiont green alga, *Oophila amblystomatia,* in the eggs and developing embryos. The *O. amblystomatia* are passed to the eggs in the oviduct of the adult (Kerney et al. 2011). These algae are thought to have a symbiotic relationship with the host salamander and benefit from the production of CO_2 and ammonia by the embryo. In turn, they produce oxygen and remove ammonia, which has been suggested to be beneficial to the embryo (Marco and Blaustein 2000). Atrazine is a photosynthetic inhibitor and it is plausible that it affects these algae. The presence of the algae appeared to contribute significantly to survival, at least under the conditions of the experiment (Olivier and Moon 2010). This study was scored relatively low due to methodological issues, including the use of a formulated product. It is possible that this salamander could be more sensitive to atrazine than other species; given its symbiotic relationship with algae, however, this is unlikely. These algae have since been isolated from eggs and tested for sensitivity to atrazine *in vitro* (Baxter et al. 2014). These data (see section on *Physiological and biochemical effects in vivo)* suggest that these algae are relatively insensitive to atrazine and that indirect effects of hatching of salamanders are unlikely at concentrations commonly found in the environment.

Reptiles

Several studies have been conducted on the effects of atrazine on the eggs of reptiles. Studies on the hatching of eggs of *Chelydra serpentina* (de Solla et al. 2006), *Alligator mississippiensis* (Gross 2001a), *Psuedemys elegans* (Gross 2001b), *Graptemys ouachitensis*, and *G. pseudogeographica* (Neuman-Lee and Janzen 2011) were conducted by exposures to solutions of atrazine in the nesting matrix. No effects on hatching were observed at maximum concentrations of atrazine ranging from 100 to 500 µg/L in a drench or 14.8 kg/ha via treated soil. A study in which Northern water snakes, *Nerodia sipedon,* were exposed to atrazine via the diet (2–200 µg/L) while pregnant, showed reduced live births but only at the median concentration tested (Neuman-Lee et al. 2013). This study was severely compromised by mortality and infections in the mothers across all treatments and control, and for this reason, the SEJ was reduced. In a study of a mixture of formulated pesticides (atrazine, dimethanamid, glyphosate, and tefluthrin) on the eggs of *C. serpentina*, there were no significant differences in hatch between control soil and those mixtures containing 1.53, 8.42, and 15.3 kg atrazine/ha (de Solla et al. 2011). Although formulations were used, this is appropriate as the matrix in which the eggs are laid is sprayed with the formulation.

Overall, there is no strong evidence for the effects of atrazine on the hatching of eggs of fish, amphibians, and reptiles. The mean score for the SOM was 2.22 ± SE 0.11, and that for relevance of responses was 0.17 ± SE 0.14 (Figure 2), and the null hypothesis was not falsified.

Time to hatching in amphibians

Time to hatching may be important to organisms in the environment if hatching is synchronized to the availability of

food for newly hatched juveniles. The null hypothesis tested in this WoE assessment was that atrazine, at concentrations commonly found in the environment, has no effect on time to hatching. Only two studies reported results for this endpoint (Rohr et al. 2004, Rohr et al. 2011) (Figure 3). These studies were conducted with the salamander *A. barbouri,* and both reported a longer time to hatch at LOEC concentrations of 400 µg atrazine/L (the score for relevance was reduced because of the exposure concentrations greater than 100 µg/L). In the 2004 study, the time to hatch varied from 6.66 to 7.29 days over the range of concentrations from 0 to 400 µg/L, which is of unknown biological significance. There were also relatively large differences in time to hatch between the two studies, despite both the studies using the same species and being conducted by the same research group. The 2004 study was conducted at 15°C and the time to hatch was 6.66 days ± 0.12, whereas, in 2011, times to hatch were 26 days at 13°C and 13 days at 19°C, with no explanation of the reason for the differences, even though the initial stages were similar in both studies and the temperature in 2004 (15°C) was an intermediate of 13°C and 19°C.

The mean score for relevance for hatching was zero and the mean score for SOM was 1.5 ± SE 0.3 (Figure 3), and is suggestive of no effects on time to hatching at environmentally relevant concentrations of atrazine. The hypothesis was clearly not falsified; however, as only two studies were found in the literature, there is some uncertainty.

Time to, or age at metamorphosis in amphibians or time to hatch in reptiles

For most amphibians, metamorphosis results in a change in diet and access to terrestrial habitat. Time to metamorphosis is important in amphibians, especially for those that are reliant on ephemeral pools during the development of the tadpole stage. Delays in metamorphosis may result in lack of survival if the pools dry up before metamorphosis is complete. Decreases in time to metamorphosis are likely less important, but could mean that terrestrial stages of amphibians might not be synchronized with their sources of food (insects). Here, the null hypothesis tested by this WoE analysis was that atrazine, at concentrations commonly found in the environment, has no effect on time to metamorphosis in amphibians or hatch in reptiles. A number of studies have characterized the effects of atrazine on time to or age at metamorphosis in amphibians (38 responses), but only one characterized time to hatch in reptiles (Figure 4).

Amphibians

No effects on timing of metamorphosis were reported in *X. laevis* at concentrations ranging from 0.01 to 400 µg atrazine/L in laboratory and semi-field studies (Carr et al. 2003, Coady et al. 2005, Du Preez et al. 2008, Hayes et al. 2002, Kloas et al. 2009a, Oka et al. 2008, Zaya et al. 2011a). One study reported effects at LOEC concentrations of atrazine of 400 µg/L or more (Zaya et al. 2011a).

Five studies reported no effects on time to metamorphosis in *L. pipiens* exposed to atrazine at concentrations ranging from 0.1 to 200 µg/L (Allran and Karasov 2000, Hayes et al. 2006b, Langlois et al. 2010, Orton et al. 2006, Relyea 2009). An increase in time to metamorphosis was reported in *Lithobates [Rana] clamitans* exposed to atrazine at 10 µg/L, but not at 25 µg/L (Coady et al. 2004). A monotonic concentration-response was not observed and the result is inconsistent with other studies in this species.

No effect on time to metamorphosis was reported in *B. americanus* exposed to concentrations ranging from 0.2 to 200 µg atrazine/L in the laboratory or in microcosms in the field (Boone and James 2003, Storrs and Semlitsch 2008, Williams and Semlitsch 2010). Similarly, no effects were reported for *Lithobates [Rana] sphenochephala* exposed to atrazine at concentrations ranging from 3 to 200 µg/L in

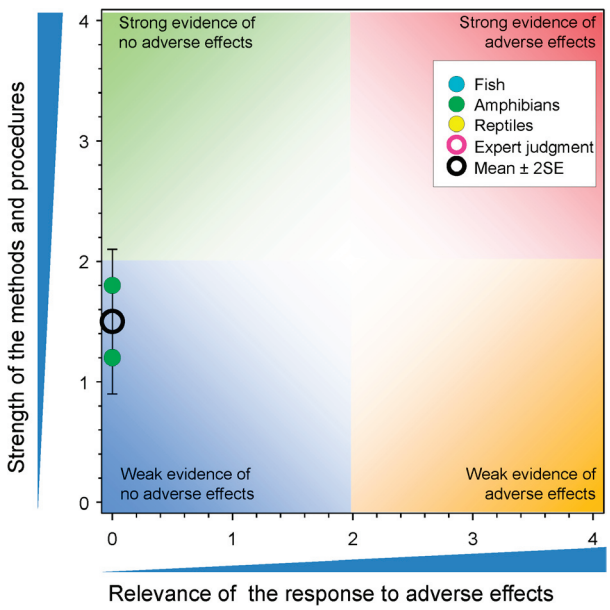

Figure 3. WoE analysis of the effects of atrazine on time to hatching in amphibians.

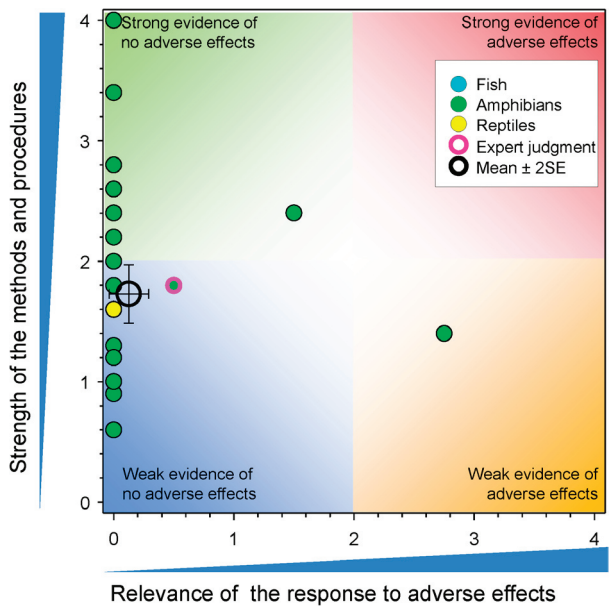

Figure 4. WoE analysis of the effects of atrazine on time to, or age at metamorphosis in amphibians or time to hatch in reptiles.

laboratory and field microcosms (Boone and James 2003, Storrs and Semlitsch 2008). In addition, no effects were reported for *Hyla versicolor* exposed to atrazine concentrations ranging from 0.2 to 100 µg/L in the laboratory and field microcosms (LaFiandra et al. 2008, Relyea 2009, Storrs and Semlitsch 2008, Williams and Semlitsch 2010).

No effect on development was reported in tadpoles of *L. sylvatica* exposed to 50 µg atrazine/L in microcosms for 4 weeks (Rohr and Crumrine 2005), or in *Limnodynastes tasmaniensis* exposed to 0.1, 1, 3, or 30 µg atrazine/L for 28 days, in the laboratory (Spolyarich et al. 2010). No effects were reported on development of *Limnodynastes raniformis* exposed to 25 µg atrazine/L for 10 weeks in the laboratory (Choung et al. 2011) or *Pseudoacris triseriata* exposed to concentrations of 0.2 and 3 µg atrazine/L in the laboratory (Williams and Semlitsch 2010).

The effects of atrazine on time to metamorphosis were studied in four species of salamander. No effects were reported in a study on *A. barbouri* which were exposed to 4, 40, or 400 µg atrazine/L (Rohr et al. 2004). A second study at different temperatures reported a delay in metamorphosis of *A. barbouri* at a LOEC concentration of 400 µg atrazine/L but only at 16°C and 19°C (not at 22°C and 25°C), and only for longer exposures (until metamorphosis vs.19 days) (Rohr et al. 2011). Exposure of *Ambystoma texanum* to an exposure concentration of 200 µg atrazine/L (> 100 µg/L) in outdoor microcosms increased time to metamorphosis by 10% (8 days) (Boone and James 2003); however, there was no effect on mass or survival. A study in *Ambystoma tigrinum* reported that exposure to an exposure concentration of 250 µg atrazine/L for 86 days increased time to stage 2 (40 days vs. 35 days), but decreased time to stage 4 (58 days vs. 60 days) (Larson et al. 1998). Larvae exposed to 75 µg/L reached stage 4 marginally later (67 days vs. 60 days) A reduced SEJ was assigned because of small effects (< 25%) and inconsistency across stages (see SI). A subsequent study in the same species reported no effects in animals exposed to atrazine at concentrations ranging from 1.6 to 160 µg/L, with and without a viral challenge (Forson and Storfer 2006). In the study on the effects of atrazine on the stage of development of *A. maculatum*, there were significant reductions at 75 days, in animals exposed to formulated atrazine at concentrations of 100, 200, and 400 µg/L (Olivier and Moon 2010). The lack of sensitivity (*in vitro*) to atrazine, of the endosymbiotic algae in the capsule of the eggs (Baxter et al. 2014, discussed above), suggests that the response reported by Oliver and Moon (2010) was not related to atrazine but to the malfunction of the incubator or other weaknesses in the experimental design. A more robust laboratory study with whole egg masses would help to resolve this question. Adverse effects were not observed in a cosm-study in juveniles of the same species exposed to 200 µg atrazine/L for 57 days (Boone and James 2003).

Reptiles

The one study on the effect of atrazine on time to hatch in reptiles (*G. ouachitensis* and *G. pseudogeographica*) reported no effects on timing following a drench application of 100 µg atrazine/L on the eggs (Neuman-Lee and Janzen 2011).

Overall, there was no strong evidence for the effects of atrazine on time to, or age at metamorphosis in amphibians or time to hatch in reptiles. The mean score for strength was 1.73 ± SE 0.12 and that for relevance was 0.13 ± SE 0.08 (Figure 4), and the null hypothesis was not falsified.

Studies with mixtures

Because it was not possible to assign causality to a single component, a study on the effects of mixtures of pesticides (including atrazine) on *L. pipiens* (Bridges et al. 2004) was not included in the analysis of WoE. A study which tested the effects of a mixture of formulated pesticides containing atrazine, glyphosate, and diphenamide on hatching in the turtle, *C. serpentina*, was also not scored for WoE. The mean day of hatching was the same as the control for mixtures containing atrazine at the equivalent of 1.53 kg/ha atrazine (3.8 and 3.9 days respectively), but was delayed for mixtures containing atrazine at 8.42 and 15.3 kg/ha (4.8 and 5.5 days respectively, P = 0.0003) (de Solla et al. 2011). Causality could not be assigned to a single mixture component in this study.

Pre-metamorphosis survival in amphibians and survival in fish, amphibians and reptiles after short-term exposure to atrazine

Survival is an apical endpoint and is obviously important in fish, amphibians, and reptiles. Here, the null hypothesis was that short-term exposure to atrazine, at concentrations commonly found in the environment, has no effect on survival. A large number of studies (137 responses) have been conducted on the effects of atrazine on pre-metamorphic survival in amphibians and sub-chronic survival in fish (Figure 5). Only two studies were conducted in reptiles. These studies generally were conducted with short-term exposure (days to a few weeks) and lethality was a primary endpoint.

Fish

Four studies on *P. promelas* (Bringolf et al. 2004, Knight et al. 2013, Mehler et al. 2008, Tillitt et al. 2010, USEPA 2005)

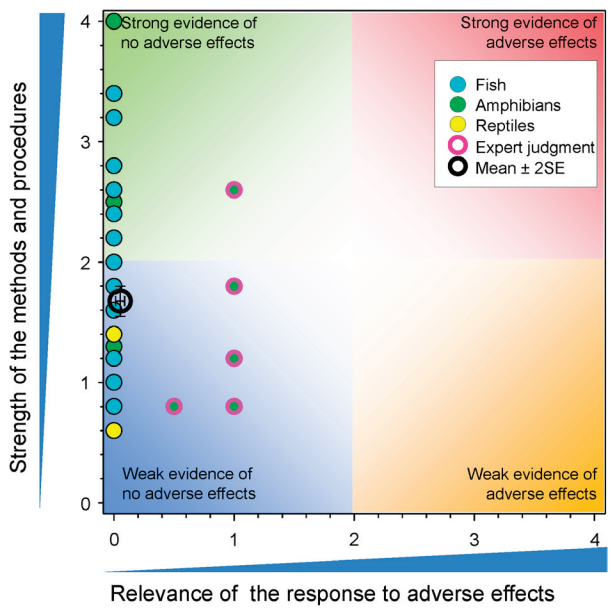

Figure 5. WoE analysis of the effects of atrazine on pre-metamorphosis survival in amphibians and survival in fish, amphibians and reptiles after short-term exposures.

showed no effects on survival after subchronic (≥ 21 d) exposures to atrazine at concentrations ranging from 0.05 to 1,000 µg/L in the laboratory or under semi-field conditions (exposed to a mixture of natural waters containing atrazine). In a full life cycle study, no effects on the survival of *P. promelas* were observed after 30- and 60-day exposure of F_0 and F_1 larvae to atrazine levels ranging from 150 to 2,000 µg/L (Dionne 1992). No mortality was observed in *Danio rerio* after exposure to atrazine at concentrations ranging from 21.6 to 10,800 µg/L for 14 days (Corvi et al. 2012), or 28-day exposures to 90 µg atrazine/L (Blahová et al. 2013, Plhalova et al. 2012) or 5-day exposure at concentrations up to 10,000 µg atrazine/L (Weber et al. 2013), or 10 and 1,000 µg atrazine/L (Schmidel et al. 2014). Acute toxicity testing with *P. promelas*, *L. macrochirus,* and *S. fontinalis* demonstrated little toxicity, with 7-d LC50 values exceeding 6,000 µg/L (Macek et al. 1976).

No effects on survival were observed in *Salmo salar* at concentrations up to 100 µg atrazine/L (Matsumoto et al. 2010, Waring and Moore 2004). No significant effects were reported in *Poecilia reticulata* exposed to 1 and 15 µg atrazine/L for 21 days (Shenoy 2012); however, the mortality rate in the controls was high, suggesting that husbandry was compromised. No effects on survival of *Prochilodus lineatus* were reported after exposures to 2, 10, and 25 µg atrazine/L for 2 and 14 days (Paulino et al. 2012a). No effects were reported in *L. macrochirus* exposed to 1,000 µg/L for 48 and 96 hours (Mehler et al. 2008), *Fundulus heteroclitus* exposed to atrazine concentrations up to 500 µg/L for 96 hours (Fortin et al. 2008), *Sciaenops ocellatus* exposed to concentrations up to 500 µg/L for 96 hours (del Carmen Alvarez and Fuiman 2005), or *Gasterosteus aculeatus* exposed to concentrations up to 100 µg/L for 42 days (Le Mer et al. 2013). Similarly, *Oncorhynchus mykiss* was unaffected by exposures of up to 555 µg atrazine/L for 4 days (Shelley et al. 2012b) and *Cyprinus carpio* exposed to up to 428 µg atrazine/L for 40 days, were unaffected (Fu et al. 2013, Wang et al. 2013). No effects were reported in *C. carpio* exposed to atrazine concentrations ranging from 5,000 to 30,000 µg/L for 96 hours (Blahová et al. 2014). No effects were reported in the Brazilian fish, *Rhamdia quelen,* exposed to atrazine at concentrations up to 100 µg/L for 96 hours (Mela et al. 2013) or 1020 µg/L for 1 or 10 days (Kreutz et al. 2012).

Several toxicity studies have been carried out in fish, but only showed effects on survival at exposure concentrations much greater than 100 µg/L. Predicted no-effect concentrations of 2,000 and 4,300 µg atrazine/L were reported for *O. mykiss* and *Ictalurus punctatus*, respectively (Howe et al. 1998). Similarly, 96-hour LC50 values for concentrations much greater than 1,000 µg atrazine/L were reported in *Oncorhynchus kisutch, O. mykiss,* and *Oncorhynchus tshawytscha,* for technical and formulated products (Wan et al. 2006). A 96-hour LC50 value greater than 42,000 µg/L was reported in *Channa punctatus* (Nwani et al. 2010, Nwani et al. 2011). An LC50 of 36,800 µg/L was reported for developing eggs of *D. rerio* (Wiegand et al. 2000). No effects on the survival of *O. latipes* were reported after 14- and 38-day exposures to up to 50 µg atrazine/L (Papoulias et al. 2014). No effects were reported in *R. quelen* exposed to 1,740 µg atrazine/L in a formulated mixture of herbicides (Koakoski et al. 2014).

Other studies reported the effects of atrazine on survival of fish at concentrations of 100 µg/L or more. Mortality (9%) was reported in *S. salar* exposed to 100 µg atrazine/L for 21 days (Nieves-Puigdoller et al. 2007), but others have not been able to repeat this observation (Matsumoto et al. 2010). Exposure of the species *R. quelen* to a concentration of 1020 µg atrazine/L for 10 days with a bacterial challenge resulted in mortality (45% to 70%) (Kreutz et al. 2010). Mortality was reported in *O. mykiss* exposed to a large concentration of 555 (but not 59) µg atrazine/L followed by a bacterial challenge (Shelley et al. 2012b).

Amphibians

In frogs, a number of studies in a variety of species were conducted. No effects were reported in *B. americanus* exposed to atrazine at concentrations of up to 201 µg/L in the laboratory or field (Allran and Karasov 2001, Boone and James 2003, Rohr et al. 2009, Williams and Semlitsch 2010). LC50 values much greater than 100 µg atrazine/L were reported in the same species in 96- and 72-hour toxicity tests (Birge et al. 1980, Howe et al. 1998). No effects were observed in an outdoor cosm study at an atrazine concentration of 10 µg/L in *H. versicolor* (Relyea 2009) and in the laboratory at up to 200 µg/L (LaFiandra et al. 2008, Williams and Semlitsch 2010). No effects were reported in stage-26 larvae of *Lithobates [Rana] catesbeianus* exposed to up to 20 µg atrazine/L for 7 days (Dornelles and Oliveira 2013).

Toxicity tests with larvae and embryos of *L catesbeianus,* showed LC50 values much greater than 100 µg atrazine/L (Birge et al. 1980, Wan et al. 2006). No effects were reported at concentrations of up to 201 µg atrazine/L in *L. clamitans* (Coady et al. 2004, Rohr et al. 2008a, Rohr et al. 2008b). The survival of *Lithobates [Rana] palustris* in field cosms treated with 117 µg atrazine/L for 4 weeks was reduced, but the LC50 values for this species in the laboratory were 17,650 µg atrazine/L or more (Birge et al. 1980), suggesting that the effects in the cosms might be due to a confounding stressor.

No effects on survival were reported at concentrations of up to 650 µg atrazine/L in *L. pipiens* (Allran and Karasov 2000, 2001, Knight et al. 2013, Orton et al. 2006, Paetow et al. 2012, Relyea 2009). Acute toxicity tests showed 96-hour LC50 values of 7,680 µg atrazine/L or more (Birge et al. 1980, Howe et al. 1998). Pre-metamorphic mortality was reported in *L. pipiens* at an exposure concentration of 300 µg atrazine/L (Koprivnikar 2010), but the larger than acceptable mortality in the controls (~ 20%) suggested that husbandry may have been compromised. There was only moderate evidence of pre-metamorphic mortality at 0.2 and 3.7 µg atrazine/L in a cosm study on *L. pipiens* (Langlois et al. 2010), but because of the large number of uncontrolled variables, it was not possible to clearly identify causality, and therefore the SEJ was reduced. In addition, this result is inconsistent with all the other reports in the literature. The study on mixtures in *L. pipiens* (Bridges et al. 2004), was not included in the WoE, because it was not possible to assign causality.

No effects were observed in *L. raniformis* exposed to 25 µg atrazine/L for 10 weeks (Choung et al. 2011). There were no effects on the survival of *R. sphenochephala* exposed to 200 µg atrazine/L in a cosm study (Boone and James 2003). No effects were reported on *L. sylvatica* at atrazine concentrations of up to 200 µg/L (Allran and Karasov

2001, Koprivnikar et al. 2007) or in the field (concentrations unknown) (Kiesecker 2002). No effects were reported in a laboratory study on *P. triseriata* exposed to atrazine at 3 µg/L from Gosner stage 25 through metamorphosis (Williams and Semlitsch 2010).

Two studies consisting of several experiments on the larvae of *Rhinella araneum* (Brodeur et al. 2009, Brodeur et al. 2013) only showed effects at concentrations greater than 1,000 µg atrazine/L.

Several studies reported no effects of exposure to atrazine at concentrations of 400 µg/L or more, and on long-term survival of *X. laevis* (Carr et al. 2003, Coady et al. 2005, Hayes et al. 2002, Kloas et al. 2009a, Langerveld et al. 2009, Lenkowski et al. 2008, Lenkowski et al. 2010, Oka et al. 2008, Tavera-Mendoza et al. 2002b, Zaya et al. 2011a). A toxicity test in this species reported 96-hour LC50 concentrations of 100,000 µg atrazine/L (Morgan et al. 1996). There is one report of the reduced survival of larvae of *X. laevis* from adult females exposed to 1 µg atrazine/L, but not from adults exposed to 10 and 25 µg/L, (Du Preez et al. 2008). Because of the inconsistent concentration-response, the SEJ was reduced (see SI).

In salamanders, no effects on survival were reported in *A. barbouri* exposed at concentrations of up to 400 µg atrazine/L (Rohr et al. 2003, Rohr and Palmer 2005, Rohr et al. 2011, larvae), in *A. texanum* and *A. maculatum,* at a concentration of 200 µg atrazine/L in field cosms (Boone and James 2003), or in *A. tigrinum* at up to 250 µg atrazine/L (Forson and Storfer 2006, Kerby and Storfer 2009, Larson et al. 1998) in the laboratory. In a study repeated in two successive years, significant reductions in short- (16 days) and longer-term (117 days) survival of larvae of *A. barbouri* were only observed in one of two years and only at a large concentration of 400 µg atrazine/L after 16 days of exposure (Rohr et al. 2004). A small (10%) decrease in the survival of virus-infected *A. tigrinum* exposed to 200 µg atrazine/L was reported, but the sample size was small and no significant interaction between the virus and exposure to pesticides was observed (Kerby and Storfer 2009). Embryos of the *A. barbouri* exposed to atrazine at concentrations ranging from 4 µg/L to 400 µg/L were reported to show reduced survival (Rohr et al. 2011). The SEJ for this response was reduced because of uncertainty in the concentrations used in the study. The response was inconsistent with other studies in the same species from the same laboratory.

Reptiles

In one of two studies on reptiles, survival of the snake *N. sipedon* was not significantly affected by exposures to atrazine concentrations of up to 200 µg/kg in the diet (Neuman-Lee et al. 2013). However, this was a methodologically weak study (SOM = 1.4) because of high mortality in the test and control animals. Survival of juvenile red-eared slider turtles (*Trachemys scripta elegans*) was not affected by exposure to *Ranavirus* and atrazine at 10 µg/L for 6 days (Polakiewicz and Goodman 2013).

Overall, some studies showed strong evidence for adverse effects on fish and amphibians in toxicity tests, but typically only where exposures were large (>> 100 µg atrazine/L). The mean score for strength was 1.68 ± SE 0.06 and that for relevance was 0.05 ± SE 0.02 (Figure 5), indicating that there is no evidence for adverse effects on pre-metamorphosis survival in amphibians and short-term survival in fish and amphibians at environmentally relevant concentrations. The null hypothesis was not falsified.

Survival of fish, amphibians, and reptiles after long-term exposures to atrazine

As above, survival is an apical endpoint with obvious importance. Here, we used the WoE analysis to test the null hypothesis that long-term exposure to atrazine, at concentrations commonly found in the environment, has no effect survival. Survival after long-term (several months) exposure to atrazine has been reported in a number of studies (14 responses) as a secondary study endpoint, and was included as a separate response because the effects were assessed over a greater time of exposure than in the studies discussed in the previous section.

Fish

Three studies report on potential effects in fish (11 responses). In *D. rerio*, no effects on long-term survival were reported after exposures to concentrations as large as 2,160 µg atrazine/L for 113 days (Corvi et al. 2012) (Figure 6). In a full life-cycle study, no effects on survival of *P. promelas* were observed after 274 days of exposure to atrazine at concentrations ranging from 150 to 2000 µg atrazine/L. (Dionne 1992). Another full life-cycle study with *P. promelas* reported no effects on survival of F_0 fish after 30- and 60-day exposures to atrazine concentrations ranging from 15 to 213 µg atrazine/L, or on F_1 fish exposed to these concentrations for 43 weeks (Macek et al. 1976). This study also reported no effects on survival in F_0 *L. macrochirus* (6- and 18-month exposures) or F_1 fish (30–90 day exposures) to 8–95 µg atrazine/L, or in F_0 *S. fontinalis* (44-week exposure to 65–720 µg atrazine/L). The survival of F_1 S. fontinalis was reduced after 60 and 90 days at exposures to concentrations

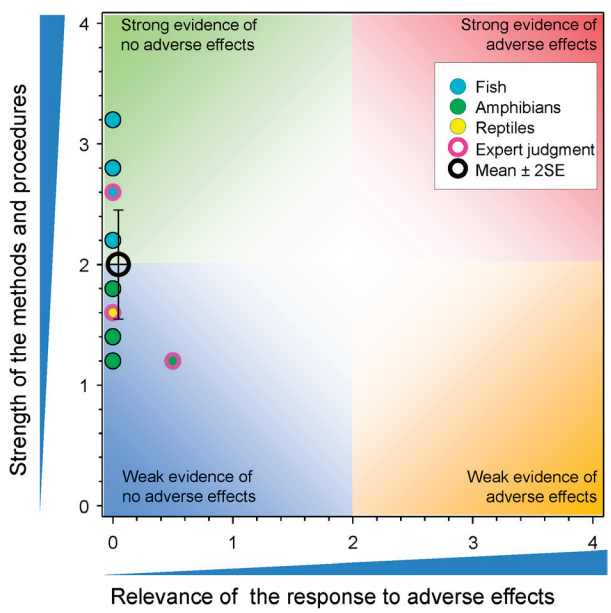

Figure 6. WoE analysis of the effects of atrazine on survival of fish, amphibians, and reptiles after long-term exposures.

ranging from 240 to 720 µg atrazine/L (>100 µg/L) (Macek et al. 1976).

Amphibians

In a study in which larvae of *A. barbouri* were exposed to atrazine at concentrations of 4, 40, or 400 µg/L, from Harrison-stage 18–28 to metamorphosis, no effects were reported on survival at metamorphosis or after one year, but a decrease was observed in year-2, but only at a concentration of 400 µg atrazine/L (Rohr et al. 2004). A follow-up study was conducted in which newly metamorphosed *A. barbouri* exposed to 4, 40, or 400 µg/L atrazine in the previous study (Rohr et al. 2004) were held for a further 410–433 days, but received no further exposure to atrazine (Rohr et al. 2006). There was a significant increase in the mortality of animals previously exposed to 40 and 400 µg atrazine/L (actual data on mortality were not provided, only standardized weighted means). Since atrazine is not bioconcentrated and has a short excretion half-life in amphibians, the responses observed were likely related to the density of the populations at the start of the study, and are inconsistent with the lack of mortality observed in the experiment in 2003, from where the animals in the 2006 study originated. The SEJ was reduced because actual mortality values were not provided, so it is not possible to assess relevance. In a further study in the same species exposed to 4, 40, or 400 µg/L atrazine from Harrison-stage 18–28 to metamorphosis, and then observed during an 11-week study period at 22°C or 27°C and wet or dry conditions, no significant effects of atrazine on survival were reported (Rohr and Palmer 2013). However, mortality in the study was large (60%–80%), probably because of poor husbandry, and the relevance of the results could not be interpreted because of poorly labeled graphs. No effects on overwinter survival of *A. maculatum* were reported 10 months after dermal exposure to an atrazine concentration of 271 µg/L for 24 hours (Mitchkash et al. 2014).

Reptiles

In reptiles, the survival at 11 months was reported to be reduced by 37% in *G. ouachitensis* and *G. pseudogeographica* following a single drench exposure to 0.1 µg/L but not to 1.0 or 100 µg/L atrazine (Neuman-Lee and Janzen 2011). A SEJ of zero was assigned because of a lack of concentration-response and because the actual species responding were not identified (the data were pooled).

Although there were a few studies on the effects of atrazine on long-term survival in fish, amphibians, and reptiles, there was little evidence of adverse effects. The mean score for strength was 2.11 ± SE 0.19, that for relevance was 0.04 ± SE 0.04 (Figure 6), and the null hypothesis was not falsified.

Successful development in fish and metamorphosis in amphibians

Development is an apical response. The null hypothesis tested in this WoE analysis was that, at concentrations commonly found in the environment, atrazine has no effect on development in fish and metamorphosis in amphibians. A number of studies (28 responses) have characterized the effects of atrazine on development in fish and amphibians (Figure 7). Development (progression from larval to juvenile stage) is an apical endpoint because a lack of proper development or changes in the rate of development can directly affect the level of the population.

Fish

No effects on growth or sexual development were reported in *P. promelas* exposed to atrazine at concentrations of up to 250 µg atrazine/L (USEPA 2005). In *S. ocellatus* exposed to 40 and 80 µg/L for 9 days, growth was reduced at 80 µg/L only (del Carmen Alvarez and Fuiman 2005). A reduced SEJ was assigned because of small differences in response (growth rate of 0.063 vs. 0.069/day; less than 10%) and the lack of effect on survival.

Amphibians

No effects on metamorphosis were observed in *X. laevis* exposed to concentrations of atrazine ranging from 0.01 to 400 µg/L (Carr et al. 2003, Hayes et al. 2002, Kloas et al. 2009a, Zaya et al. 2011a). A decrease in the rate of metamorphosis in the same species exposed from stages 47 to 62 was observed in two experiments, but only at an exposure concentration of 400 µg atrazine/L (Zaya et al. 2011a). No effects were observed in *L. pipiens* exposed to 20 and 200 µg atrazine/L (Allran and Karasov 2000) or in *L. clamitans* at 10 or 25 µg/L (Coady et al. 2004). No effects on metamorphosis were observed in *H. versicolor* exposed to 20 and 200 µg atrazine/L in the absence of predator-stress (LaFiandra et al. 2008); however, the proportion initiating metamorphosis was reduced in tadpoles exposed to a predator as well as 200 µg atrazine/L.

No effects on time to metamorphosis were observed in *Rhinella arenarum* exposed to concentrations of up to 1,000 µg atrazine/L from stage 42 to metamorphosis (Brodeur et al. 2013). In other experiments, shorter times to reach stage

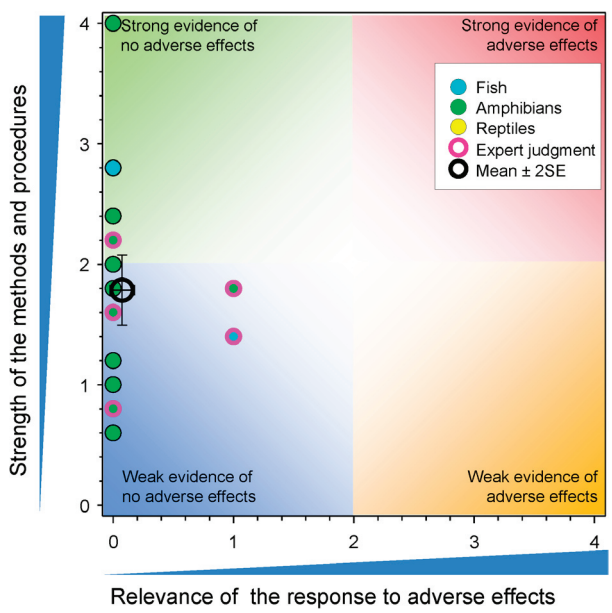

Figure 7. WoE analysis of the effects of atrazine on successful development in fish and metamorphosis in amphibians.

42 from stage 39 and from stage 25 to 39 were observed in *R. arenarum,* but only at an exposure concentration of 1,000 µg atrazine/L (Brodeur et al. 2009, Brodeur et al. 2013). In another experiment on the same species, reduced time to metamorphosis was observed at atrazine concentrations of 1, 10, and 100 µg/L but not at 0.1 and 1,000 µg atrazine/L (Brodeur et al. 2013). A SEJ of zero was assigned because there was no effect on the number of tadpoles metamorphosing, shorter times to metamorphosis were not considered adverse, and there were no reductions in weight, which was actually significantly increased at 1 and 10 µg atrazine/L.

Effects on early and late stage larvae of *L. pipiens, L. clamitans, B. americanus,* and *L. sylvatica* were reported after exposures to 3, 30, and 100 µg atrazine/L (Storrs and Kiesecker 2004), but a reduced SEJ was assigned because of lack of clarity in the statistical analysis and inconsistent and "counterintuitive concentration-responses" (see SI). The response was also inconsistent with reports of lack of effects in the same species at greater concentrations of atrazine in other laboratories (see above). A reduction in the number of *L. pipiens* reaching metamorphosis in cosms treated with 3.7 µg of formulated atrazine/L was reported (Langlois et al. 2010), but this is inconsistent with the lack of response observed by others (Allran and Karasov 2000). Effects were reported in *L. raniformis* exposed to 25 µg/L in a preliminary test (Choung et al. 2011), but, as the definitive test showed no differences in this response, an SEJ of zero was assigned. Time to developmental stage was reported to be affected in tadpoles of *O. septentrionalis* exposed to 66 µg atrazine/L for 6 days in field cosms (Rohr et al. 2013). In another study on the same species, no effects of exposure to 102 and 204 µg atrazine/L for 28 days were reported (Halstead et al. 2014). The score for relevance was reduced because of high mortality in treated and control cosms, indicating problems with husbandry.

Overall, for studies examining effects on development and metamorphosis, the mean score for strength was 1.79 ± SE 0.15, while that for relevance was 0.07 ± SE 0.05 (Figure 7) and the null hypothesis was not falsified.

Weight or size of fish, amphibians, and reptiles

Size and weight are responses that are related to the apical endpoint of growth. This WoE analysis tested the null hypothesis that atrazine, at concentrations commonly found in the environment, adversely affected weight and/or size. A number of studies (122 responses) have reported the effects of atrazine on weight and the correlated endpoint of size. These are summarized in Figure 8. Loss of weight or reduced rate of weight-gain is often used as an indicator for generalized effects of chemicals in toxicity testing of mammals (Ramsingh 2010). There can be implications at the population-level if, for example, smaller individuals are less fecund than their more typical-sized counterparts.

Fish

Three studies reported no effects of atrazine on size and/or weight of fathead minnows at concentrations up to 250 µg atrazine/L (Bringolf et al. 2004, Knight et al. 2013, USEPA 2005). In a full life-cycle study, effects on size or weight of

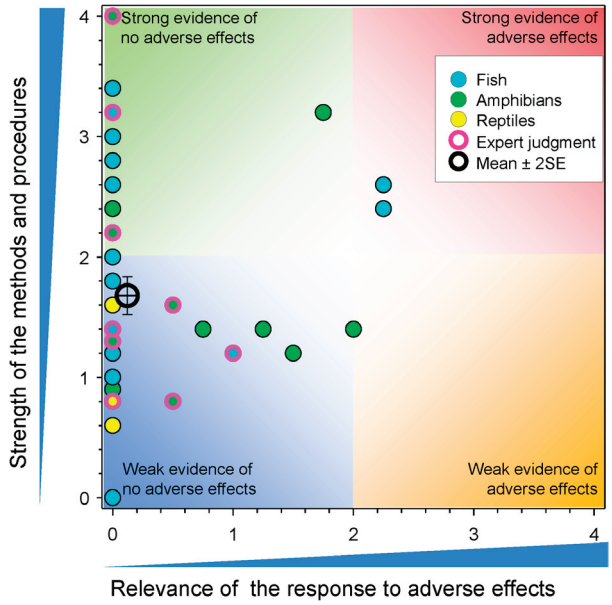

Figure 8. WoE analysis of the effects of atrazine on weight or size of fish, amphibians, and reptiles.

P. promelas were observed for the F_0 at 60 days and F_1 larvae at 30 days, and in the F_0 at study termination (274 days). These effects on size were observed at exposures to atrazine at concentrations of 460 µg/L or greater (Dionne 1992). Full life-cycle studies reported no effects on lengths or weight of F_0 *P. promelas* (9–43 weeks, 15–213 µg atrazine/L exposures) or F_0 *L. macrochirus* (6–18 months, 8–95 µg atrazine/L exposures), and no effects of these same concentrations on the lengths of the F_1 generations after 30–60-day- (*P. promelas*) or 30–90 day- (*L. macrochirus*) exposures (Macek et al. 1976). These authors report greater sensitivity in *S. fontinalis*, with the length and weight of F_0 fish reduced (90 day-exposure to 240–720 µg atrazine/L; 306 day-exposure to 120–720 µg atrazine/L) as well as a reduction in these metrics in F_1 fish (90 day-exposure to 240–720 µg atrazine/L) (Macek et al. 1976).

The length of *S. ocellatus* was unaffected at 40 and 80 µg atrazine/L (McCarthy and Fuiman 2008). No effects on length or residual mass were reported in *F. heteroclitus* exposed atrazine at concentrations ranging from to 5 to 500 µg/L for 96 hours (Fortin et al. 2008), but condition factor was reduced at a concentration of 500 µg atrazine/L. No effects on mass of *S. salar* were reported at concentrations up to 100 µg atrazine/L (Matsumoto et al. 2010, Nieves-Puigdoller et al. 2007) or on length (Nieves-Puigdoller et al. 2007). No effects on length were reported in *D. rerio* exposed from fertilization to 72 hours to concentrations up to 30 µg atrazine/L. The length and weight were reduced by 5% and 20% respectively in *D. rerio* exposed atrazine at 216 µg/L or greater, for 113 days (Corvi et al. 2012). Reductions in the specific growth rate (1.4 vs. 1.7%/d) (Blahová et al. 2013) and growth rate (17%) (Plhalova et al. 2012) were reported in the same species exposed to atrazine at a concentration of 90 µg/L but not at 0.3, 3 or 30 µg/L. No effects on mass or length were observed in *Lates calcarifer* exposed to concentrations of atrazine ranging from 0.1 to 100 µg/L for 48 hours (Kroon et al. 2014).

A field study on *Micropterus dolomieu* showed increased weight (males) and increased weight and length (females) at

sites with greater average concentrations of atrazine (Iwanowicz et al. 2009). This was not deemed an adverse effect and a reduced SEJ was assigned. Reductions in mass protein content and rate of protein synthesis of larvae of *S. ocellatus* exposed to 40 and 80 µg atrazine/L were reported (McCarthy and Fuiman 2008), but the results showed inconsistent statistical significance and were assigned a reduced SEJ.

Exposure of *G. aculeatus* to concentrations up to 100 µg atrazine/L for 42 days caused no effects on the condition factor except for a small decrease (0.76 vs. 0.83) in one of two repeated studies and only at an intermediate concentration (10 µg/L) (Le Mer et al. 2013). A reduced SEJ was assigned because of inconsistency and lack of concentration-response. A study on Tilapia, *Sarotherodon mossambicus [Oreochromis mossambicus, Tilapia mossambica],* reported reduced weight at a concentration of 1100 µg/L for up to 90 days (Prasad and Reddy 1994). No effects on final weight or tank biomass were reported in *R. quelen* exposed to 1,740 µg atrazine/L in a formulated mixture of herbicides (Koakoski et al. 2014).

Amphibians

A large number of studies have reported on the effects of atrazine on the weight and/or size of amphibians. No significant effects on size and/or weight were reported for *X. laevis* exposed to concentrations ranging from 0.01 to 100 µg atrazine/L in laboratory and field studies (Carr et al. 2003, Coady et al. 2005, Du Preez et al. 2008, Hayes et al. 2002, Kloas et al. 2009a, Smith et al. 2005, Tavera-Mendoza 2001, Zaya et al. 2011a). No differences in the weight of male *X. laevis* from exposed and control sites were observed in a field study (Du Preez et al. 2005a). Likewise, no effects were reported in *L. clamitans* exposed to 10 and 25 µg/L (Coady et al. 2004), *L. pipiens* exposed to concentrations ranging from 0.2 to 200 µg atrazine/L (Allran and Karasov 2000, Knight et al. 2013, Langlois et al. 2010, Orton et al. 2006, Paetow et al. 2012, Relyea 2009), *L. sylvatica* exposed to 50 µg/L (Rohr and Crumrine 2005), *L. tasmaniensis* exposed to 0.1, 1, 3, or 30 µg atrazine/L (Spolyarich et al. 2010), or *H. versicolor* exposed to atrazine at concentrations ranging from 0.2 to 200 µg/L (LaFiandra et al. 2008, Williams and Semlitsch 2010). Similarly, no effects were reported in *L. raniformis* exposed to 25 µg atrazine/L for 10 weeks (Choung et al. 2011) or in *B. americanus* and *P. triseriata* exposed to 0.2 and 3 µg/L for 27–29 days (Williams and Semlitsch 2010). No effects of exposures to up to 20 µg atrazine/L for 7 days were reported for stage-26 larvae of *L catesbeianus* (Dornelles and Oliveira 2013).

Reductions in organ weight, body weight, and size of *X. laevis* tadpoles exposed to large exposure concentrations ($\geq$200 µg atrazine/L) were reported in several studies (Langerveld et al. 2009, Zaya et al. 2011a). Mass of *L. sphenochephala* and *B. americanus* exposed to atrazine in a cosm study for 57 and 80 days, respectively, was reduced, but only at an exposure concentration of 200 µg/L (Boone and James 2003).

In contrast, some studies have reported adverse effects in frogs. Mass and developmental stage of *L. pipiens* exposed to 3 µg atrazine/L were reported to be reduced (Koprivnikar 2010), but because of high mortality in the controls, the SEJ was reduced. A decrease in body weight and length of less than 10% was reported in *L. pipiens* exposed to 0.1 µg atrazine/L (Hayes et al. 2006b), but only one concentration was tested. Others have not observed this response in the same species (as described above). Greater length and body condition were observed in *R. arenarum* exposed to atrazine at concentrations greater than 1 µg/L (Brodeur et al. 2013), but as these increases are not considered adverse, a SEJ of zero was assigned. A similar observation was made in *H. versicolor* exposed to 10 µg atrazine/L in a cosm where mass increased (Relyea 2009), and a SEJ of zero was assigned. A field study reported that the mass of *L. sylvatica* was reduced at sites subjected to agricultural inputs (Kiesecker 2002); however, as concentrations of atrazine were not measured, it was not possible to assign causality and the SEJ was reduced. The body mass of female *X. laevis* exposed in the field to different concentrations of atrazine ranging from 1.2 to 9.3 µg atrazine/L was smaller than controls (Du Preez et al. 2005a). The reasons for this were not clear but may have been related to the timing of oviposition in relation to the time of collection. A study on *L. pipiens* exposed to one concentration (2.1 µg/L) and then challenged over 74 days with the fungus *Batrachochytrium dendrobatidis (Bd),* showed a decrease in weight gain (~9%) but no differences in snout-vent length (SVL) (Paetow et al. 2012). No effects were reported on the mass of tadpoles of *O. septentrionalis* exposed for to up to 106 µg atrazine/L and a *Bd* challenge for 28 days (McMahon et al. 2013), but in a cosm study in the same species exposed to 66 µg atrazine/L for 6 days, effects were reported (Rohr et al. 2013). The score for relevance was reduced because of high mortality in treated and control cosms, indicating problems with husbandry.

For salamanders, no effects were reported in *A. barbouri* exposed to 4, 40, or 400 µg atrazine/L (Rohr et al. 2003), *A. tigrinum* exposed to up to 160 µg/L with and without a viral challenge (Forson and Storfer 2006), or *A. texanum* exposed to 200 µg/L in a cosm (Boone and James 2003). The length at metamorphosis of larvae of *A. barbouri* exposed to a concentration of 400 µg atrazine/L from Harrison-stage 18–28 to metamorphosis was reduced by small amounts (0.7 mm to 1 mm; 2% and 4%) (Rohr et al. 2004). The weight and length of stage 2 larvae of *A. tigrinum* exposed to atrazine at 250 µg/L for 86 days was reduced by less than 10% (Larson et al. 1998). In experiments on *A. barbouri* exposed to 4, 40, or 400 µg/L atrazine to metamorphosis and then observed as single animals (singlicates) or triplets, on days 137 and 239 after the last exposure to atrazine, the animals showed small ($\leq$10%) reduction in weight when exposed to increased temperatures (Rohr and Palmer 2005 905, 2013). The mechanism for this was not characterized, so the cause of the response is not clear, but there was high mortality (60%-80%) in the later study (Rohr and Palmer 2013), which resulted in the assignment of a SEJ of zero. No effects were reported on the weight and SVL of *A. maculatum* after dermal exposure to a concentration of 271 µg/L for 24 hours (Mitchkash et al. 2014).

Reptiles

In studies on reptiles, no effects were reported on the weight of the residue remaining in the eggs after hatching of *C. latirostris* treated with 200 µg atrazine/kg (Beldomenico et al. 2007). Similarly, no effects on length at hatching and mass at hatching or 11 months later were observed in the turtles *G. ouachitensis* and *G. pseudogeographica*, following a single drench exposure of the eggs to atrazine at 0.1, 1.0, or 100 µg/L (Neuman-Lee and Janzen 2011). A study on the effects of mixtures of compounds (containing 1.53, 8.42, and 15.3 kg atrazine/ha) in *C. serpentina* showed no differences in weights of hatchlings (de Solla et al. 2011). The mass, length, and width of hatchlings of the Red-eared slider turtle (*T. s. elegans*) exposed to atrazine at a level of 0.5 µg/kg in the egg, were increased when incubated at 26°C but not at 29.2°C (Willingham 2005). The SEJ was reduced after expert review because the effect, i.e., increase in weight, was not judged to be adverse. No effects on weight or length were reported in the same species after exposure to 6 µg atrazine/L for 6 days (Polakiewicz and Goodman 2013). A loss of weight during the incubation of eggs and the weight of hatchlings of *C. latirostris* treated topically with a large nominal dose of 200 µg atrazine/kg and incubated at a male-producing temperature was reported (Beldomenico et al. 2007).

Overall, there was no strong evidence of any adverse effects of atrazine on weight and/or size of fish, amphibians, and reptiles (Figure 8). The mean score for strength was 1.68 ± SE 0.08, and that for relevance was 0.12 ± SE 0.04. Several strong studies reported no effects, and the null hypothesis was not falsified.

Deformities in fish, amphibians and reptiles

Deformities (externally visible malformations) can prevent the completion of development or make organisms less competitive, with reduced ability to survive in the wild, and are thus important responses that relate to the apical endpoint of survival. The null hypothesis for this WoE assessment was that exposure to atrazine, at concentrations commonly found in the environment, does not cause deformities. There were 27 response for this endpoint (Figure 9).

Fish

One study (3 responses) on development in zebrafish (*D. rerio*) exposed to atrazine at concentrations of up to 30 µg/L from 1 hour to 72 hours post-fertilization reported no effects on gross deformities of the body, head, or eye (Weber et al. 2013). Developmental anomalies were reported in the developing eggs of *D. rerio* at very large concentrations of atrazine (≥ 5,000 µg/L (Wiegand et al. 2001).

Amphibians

Several studies showed no effects of atrazine on deformities (e.g., missing limbs or digits, hind limb length, development of the gut) (Figure 9); gonadal malformations are reported separately. No deformities were observed in *X. laevis* exposed to concentrations ranging from 0.1 to 100 µg atrazine/L (Du Preez et al. 2008, Tavera-Mendoza 2001, Wolf 2012),

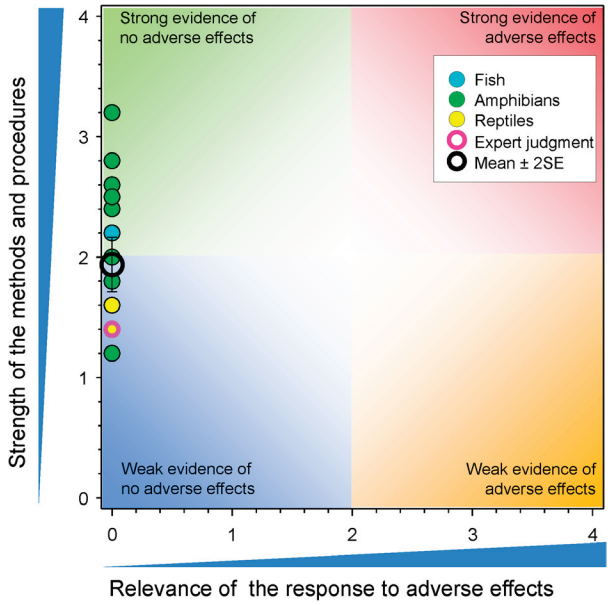

Figure 9. WoE analysis of the effects of atrazine on deformities in amphibians and reptiles.

in *L. tasmaniensis* exposed to 0.1, 1, 3, or 30 µg atrazine/L (Spolyarich et al. 2010), in *L. tasmaniensis* and *Limnodynastes fletcheri* exposed to atrazine at concentrations ranging from 0.1 to 0.7 µg atrazine/L in the field, or in *L. clamitans* exposed to atrazine at concentrations ranging from 0.17 to 250 µg/L in the field (Murphy et al. 2006b). No deformities were observed in the salamanders *A. barbouri* and *A. maculatum* exposed to a range of concentrations from to 4 to 400 µg atrazine/L and from 50 to 400 µg atrazine/L, respectively (Olivier and Moon 2010, Rohr et al. 2003).

Three studies reported deformities (e.g., malformations in embryos, cranial muscle, heart, or gut tissue) in *X. laevis* (Lenkowski et al. 2008, Lenkowski et al. 2010, Morgan et al. 1996), but all of these were only observed at exposure to concentrations of 350 µg atrazine/L or greater. Similarly, deformities were reported in FETAX-type tests (ASTM 1992) with *L. pipiens, B. americanus,* and *L. sylvatica,* but the effects were only observed at atrazine concentrations over a range of 200 to 1100 µg atrazine/L (Allran and Karasov 2001).

Reptiles

No deformities were observed in turtle hatchlings from eggs exposed to drenches of 100 µg atrazine/L or less (Neuman-Lee and Janzen 2011). Compared to the control, greater asymmetry of scale rows in the mid-body and in the jaws were observed in still-born offspring of the snake *N. sipedon* exposed to atrazine via the mother's diet containing 2, 20, and 200 µg atrazine/kg (Neuman-Lee et al. 2013). However, this study was weak (SOM = 1.4) and was confounded by poor survival and high maternal mortality (see SI), which may have been the root cause of the observed effects (SEJ was reduced). A study on the effects of mixtures of compounds (containing 1.53, 8.42, and 15.3 kg atrazine/ha) in *C. serpentina* showed no differences in the incidence of deformities between control and treated eggs (de Solla et al. 2011).

Overall, the mean score for strength was 1.96 ± SE 0.12, and that for relevance was 0.00 ± SE 0.00. Overall, there is no evidence to suggest that atrazine causes deformities in developing amphibians or reptiles at environmentally realistic concentrations (Figure 9), and the null hypothesis was clearly not falsified.

Direct effects of atrazine on sexual differentiation and development

A number of studies have characterized the effects of atrazine on sexual differentiation and gonadal development. The biological responses range from changes in the sex ratio and gonadal gross morphology, such as visible abnormalities and size of the gonads, to histopathological changes at the tissue level. Most of these studies have focused on the male gonads, probably in response to the attention given in early studies on amphibians. Many of these studies have been the subject of regulatory review internationally, and by the USEPA's SAP.

Sex ratios in fish, amphibians, and reptiles

Sex ratio is a potentially important characteristic of populations, and alterations in this value might have consequences for the apical endpoints of reproduction and the sustainability of populations. The null hypothesis tested in this WoE analysis was that atrazine, at concentrations commonly found in the environment, does not cause alteration of sex ratios. In addition to reports of partial feminization in fish, amphibians, and reptiles (see section on *Gonadal abnormalities in the testes*), some authors have reported that atrazine causes complete feminization of males during sexual differentiation, thereby affecting the normal sex ratio, which is nominally 50/50, male/female. The proposed, but scientifically unsupported mode of action, is an up-regulation of the aromatase enzyme by atrazine (see section on *Expression or activity of aromatase*), resulting in reduced testosterone and increased estrogen (see section on *Concentrations of steroid hormones*). The effect of steroidal hormone treatments during sexual differentiation on sex ratios has been well documented in fish (Jobling et al. 2009, Pandian and Sheela 1995), amphibians (Hayes 1998, Olmstead et al. 2009), and reptiles (Bull et al. 1988, Dorizzi et al. 1994, Rhen and Lang 1994). The strength and relevance of the studies on the effects of atrazine on sex ratio (40 responses) are summarized in Figure 10. When characterizing sex ratios, we recognized that the determination of sex ratio, especially where small numbers of animals were used in the tests, can be confounded by natural variability. This was considered in the WoE analysis.

Fish

In methodologically robust studies repeated in 2007 and 2008 (SOM = 3.2), no effects on sex ratio were observed in *G. aculeatus* exposed to atrazine at concentrations ranging from 0.1 to 100 µg/L, from hatching to 42 days (Le Mer et al. 2013). Another study reported an increase in the frequency of female *D. rerio* after exposure from 17 days post-fertilization to 6 months, to concentrations of 21.7, 217, and 2,170 µg atrazine/L (Suzawa and Ingraham 2008). The effect was reported for all concentrations; however, no statistics were provided. This study had a number of weaknesses and uncertainties, and

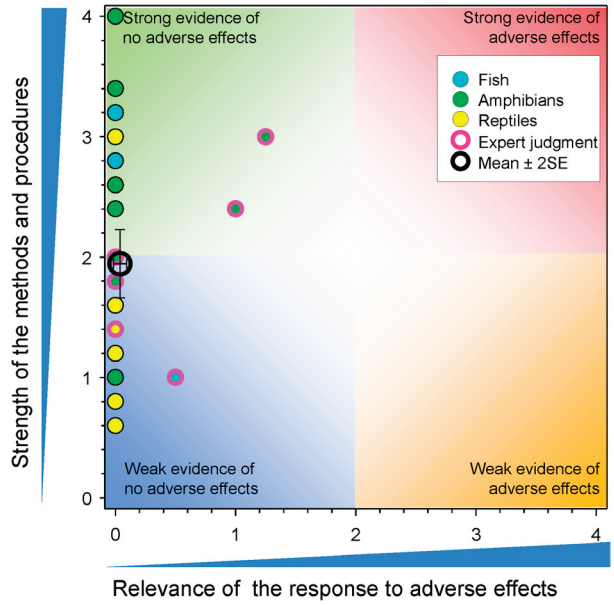

Figure 10. WoE analysis of the effects of atrazine on sex ratio in fish, amphibians and reptiles.

therefore, scored low for strength of methods (SOM = 1.0) (see SI).

In contrast to the study by Suzawa and Ingraham (2008), Corvi et al. (2012) reported different findings. They used similar concentrations (0, 21.6, 216, and 2,160 µg/L, of technical grade atrazine) and a similar duration of exposure as in the study by Suzawa and Ingraham (2008), but the study was much stronger (SOM = 2.8) with respect to identification of fish strain, replication, use of a positive control, verification of exposure concentrations, blind histology, and availability of all raw data. The study was repeated in 2009 and 2010, and in each year, large and significant shifts towards females were observed in the E2 positive controls, but not in any of the atrazine-treated groups.

Amphibians

A large number of studies of varying SOM (SOM ranging from 1–4) have examined sex ratios in frogs. All but one study (Zaya et al. 2011a) were previously discussed in the section on *Direct effects of atrazine on development*. No significant effects on sex ratio were reported in *X. laevis* in a South African field study (Du Preez et al. 2005a), an outdoor microcosm (Jooste et al. 2005), or in multiple laboratory studies which exposed frogs to concentrations of atrazine ranging from 0.01 to 100 µg/L, during the sexual differentiation phase (Carr et al. 2003, Coady et al. 2005, Du Preez et al. 2008, Kloas et al. 2009a, Tavera-Mendoza et al. 2002b, Zaya et al. 2011a). In the publication by Zaya et al. (2011a), the results of multiple studies are reported. No effect on sex ratio was found when *X. laevis* was exposed from Nieuwkoop and Faber (NF) stage 47 to 62 to 400 µg atrazine/L (2 studies), to 200 and 400 µg atrazine/L (2 studies), or to 25 and 200 µg atrazine/L (1 study). These studies focused primarily on metabolic endpoints and scored low for strength (SOM = 0.6–1.2). It should also be noted that although Jooste et al. (2005) reported no significant differences between treatments, the percentages of females

were 55, 52, 61, and 53 in the control and when treated with 1, 10, and 25 µg atrazine/L, respectively. However, when we reanalyzed the data on the basis of an expected ratio of 50%, a significant female-biased ratio at 10 µg/L (X^2, $p < 0.01$) was observed. There was no concentration-response and the differences in ratio were modest; however, the SEJ for the relevance of this response was increased.

In two studies reported by Storrs-Mendez and Semlitsch (2010), the authors found no effect on the sex ratio of *L. sphenochephala* exposed to 1, 3, and 30 µg atrazine/L from Gosner stage 25 to metamorphosis, or of *L. sphenochephala* and *H. versicolor* exposed to 1 or 10 µg atrazine/L from Gosner stage 25 to four months of age. LaFiandra et al. (2008) also found no effect on sex ratio of *H. versicolor* exposed to atrazine at concentrations of 0, 20, or 200 µg/L, from Gosner stages 25 to 42, in the presence and absence of a nonlethal predator. Two other species of Ranids (*L. pipiens* (Orton et al. 2006) and *L. clamitans* (Coady et al. 2004)) were examined after exposure to concentrations ranging from 10 to 25 µg atrazine/L during sexual development, with no effects on sex ratio reported. In studies with Australian amphibians, no shift in sex ratio was observed in *L. tasmaniensis* exposed to 0.1, 1, 3, or 30 µg atrazine/L for 7, 14, 21, and 28 days from Gosner stages 28 to 42 (Spolyarich et al. 2010), or in field-collected *L. tasmaniensis*, *L. fletcheri,* or *L. raniformis* (Spolyarich et al. 2011). A study on the effects of mixtures of herbicides on sex ratios in *Lithobates blairi* from wetlands in Nebraska reported no correlation with concentrations of atrazine (Papoulias et al. 2013). However, the frogs were exposed to a mixture of atrazine and other herbicides, and the concentrations were measured at the time of collection, not during development (WoE analysis was not conducted because causality could not be assigned).

Three publications did report effects on the sex ratio of amphibians. Storrs-Mendez and Semlitsch (2010) conducted two sets of experiments. The first monitored the development of tadpole larvae from Gosner stage 25 to metamorphosis, at 3-week intervals, with exposure concentrations of 1, 3, or 30 µg atrazine/L, and the second examined juvenile gonads at metamorphosis, after exposure to 1 and 10 µg atrazine/L. These authors reported significant differences in sex ratio when control ratios were compared to atrazine-treated larval *H. versicolor* and for both larval and juvenile *B. americanus*. The reported effects may have been the result of too small a sample size. Since the sex of the organisms are unknown at the time of initiation of the study, adequate numbers of animals are needed to ensure that starting ratios are approximately 50/50, males to females. Olmstead et al. (2009) recently illustrated the importance of sample size in accurately detecting shifts in sex ratios. The Monte Carlo analysis simulated the effects of 1,000 different virtual experiments, each of which contained 30 tanks with 30 individual frogs per tank. In addition, varying numbers of individuals per tank and replicates of tanks were also simulated. This analysis shows that even with 10 individuals replicated 3 times (30 individuals), the likelihood of accurately detecting a 20% effect on sex ratio is only approximately 20%. Storrs-Méndez and Semlitsch (2010) indicate a design of 1 individual per "canning jar", replicated 30 times; however, the animals were removed at various time points, reducing the actual sample size to as low as 5. A sample size of five to ten animals with no replication was not simulated by Olmstead et al. (2009) but clearly the power to accurately detect real and significant shifts in sex ratios with this sample size is negligible.

Other studies also underscore the need for adequate sample sizes to accurately determine effects on sex ratios. A significant decrease in the number of male tadpoles of *L. pipiens* was reported after exposure to 3.7 µg formulated atrazine/L in outdoor microcosms (Langlois et al. 2010). However, a closer examination revealed that 62% of the 60 control frogs were males by chance. Other authors report sex ratios closer to 1/1 ($54.0 \pm 2.8\%$ males) (Chen et al. 2009), and a study by Hogan et al., conducted in the same laboratory as that by Langlois et al. (2010), reported ratios ranging from 1/1 to 1/0.8 (Hogan et al. 2008). Again by chance, and despite a much smaller number of frogs (31) in the group treated with 3.7 µg atrazine/L than in the controls, the proportion of males in the atrazine-treated group (42% males) was actually closer to the expected ratio than to the controls. Due to this weakness, the overall evaluation of relevance (SEJ) was reduced. A laboratory study with *X. laevis* also suffered from low sample sizes and random initial sex ratios (Oka et al. 2008). The authors reported significant increases in the number of female metamorphs of *X. laevis* exposed to concentrations of 10 and 100 µg atrazine/L, from NF stages 49 to 66, but not with concentrations 0.1 or 1 µg atrazine/L. However, only about 39% of the controls were females ($N = 85$), making comparison less reliable. Furthermore, mortality was observed in some atrazine-treated groups, creating unequal replicates, and further reduced sample sizes ($N = 30 – 60$). The overall evaluation of relevance (SEJ) was therefore reduced.

Reptiles

A number of studies have examined the potential effects of atrazine exposure during reptilian embryo development on sex ratios. The treatments were generally topical to the eggs (often with a solvent), or via drenches to the soil or nesting material. Since sex ratios in reptiles are temperature-dependent, studies typically tested at male- and/or female-producing temperatures for comparison to controls. No effect on the sex ratio of hatching turtles was observed in eggs of *C. serpentina* exposed to formulated atrazine at 1.48 and 14.8 kg/ha in soil (de Solla et al. 2006), in *P. elegans* with drench exposure to atrazine at concentrations of 10, 50, 100, and 500 µg/L (Gross 2001b), in *P. elegans* exposed to 0.5 µg/kg (Willingham 2005), or in *G. ouachitenisis* and *G. pseudogeographica* following exposure to 0.1, 10, or 100 µg atrazine/L (Neuman-Lee and Janzen 2011).

Several studies have also been conducted in caimans and alligators, with no effects on sex ratio. In separate studies, Beldomenico et al. (2007) and Rey et al. (2009) topically treated the eggs of *C. latirostris* with 200 µg formulated atrazine/kg at male-producing temperatures, with no effects. In a similar study, (Stoker et al. 2008) treated eggs of this species with the same atrazine dose, but at female-producing temperatures, and also observed no effect on sex ratio. Eggs of *A. mississippiensis* were topically treated with 0.14, 1.4, and 14 mg atrazine/kg and no effect on hatching sex ratio was observed after incubation, at either male- or female-producing temperatures (Gross 2001a).

One study on snakes reported a decreased proportion of female offspring from adult female *N. sipedon,* exposed via a fish diet to 2, 20, and 200 µg atrazine/kg for 3 months (Neuman-Lee et al. 2013). Proportionally fewer females were born with exposure to atrazine at 200 µg/L, but this was not significant. However, this study suffered from a number of serious weaknesses related to sample size, significant mortality in the control (43%), lethal fungal infections across all treatments, and issues related to husbandry (see SI). For these reasons, the overall relevance score (SEJ) was reduced.

The mean score for strength was 1.95 ± SE 0.14, while that for relevance was 0.04 ± SE 0.03, and the null hypothesis was not falsified. There was virtually no evidence of effects of atrazine on sex ratio in several species of reptiles, although the SOM for many of these studies was rather low. In fish, one study provided weak evidence of no effect, while two very strong studies provided strong evidence of no effect. Across many species of amphibians, approximately 80% of the responses indicated no effects on sex ratios, over half of which came from very strong studies (SOM ≥ 2). The few studies reporting effects on sex ratio in amphibians tended to be weakened by a poor sample size and atypical sex ratios in the controls. Overall, as indicated by the individual scores in Figure 10, there is very little evidence that is supportive of the effects of atrazine on sex ratios in fish, amphibians, and reptiles.

Gonad-somatic index in fish and amphibians

The gonad-somatic index (GSI) is an expression of the weight of the gonad relative to the total body weight of the animal, and is therefore a potential indicator of sexual development or abnormalities. For example, an animal with an underdeveloped gonad relative to its size or weight may suggest a degree of reproductive impairment. GSI will also vary with the sexual maturity and the reproductive state of the animal. The null hypothesis for this WoE analysis was that atrazine, at concentrations commonly found in the environment, does not cause changes in the gonad-somatic index. WoE scores for these responses (30) are summarized in Figure 11.

Fish

The GSI has been examined in both males and females of 5 species of fish, with most exhibiting no response to atrazine exposure. No effects on GSI were observed in *P. promelas* (Bringolf et al. 2004, Knight et al. 2013, Tillitt et al. 2010, USEPA 2005), *Carassius auratus* (Nadzialek et al. 2008, Spanó et al. 2004), *S. salar* (Matsumoto et al. 2010), or male and female *O. latipes* (Papoulias et al. 2014). Exposure concentrations across these studies ranged from 0.5 µg to 1,000 µg atrazine/L and study durations were of up to 56 days. A physiological seawater challenge study with *S. salar* parr reported significantly reduced GSI in males but not in females (Nieves-Puigdoller et al. 2007). Finally, a field study was conducted over a 2-year period to investigate the potential impacts of environmental pollutants (including atrazine) on sexually mature *M. dolomieu* (Iwanowicz et al. 2009). Fish were collected above and below wastewater treatment plants (WWTPs), and it was found that the GSI was increased in both males and females at the upstream site relative to the downstream site.

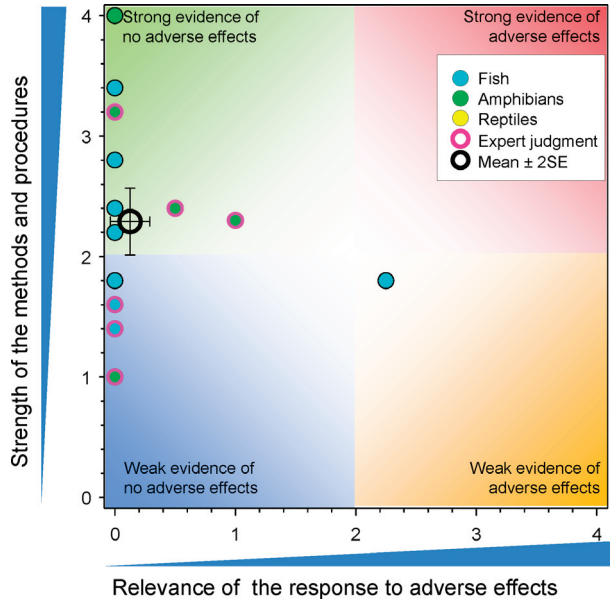

Figure 11. WoE analysis of the effects of atrazine on gonadal-somatic index in fish and amphibians.

The concentrations of atrazine and metabolites were doubled at the upstream sites compared to those downstream. However, other compounds were present in the water, so causality could not be established (see SI).

Amphibians

GSI has been examined in males and females of four species of amphibians. Laboratory studies with *L. pipiens* and *X. laevis*, at concentrations ranging from 0.01 to 1,000 µg/L, reported no effects on the GSI (Kloas et al. 2009a, Knight et al. 2013). One laboratory study reported differences in the GSI of adult male *X. laevis* exposed to atrazine at 10 µg/L but not at 100 µg/L (Hecker et al. 2005). The SEJ was reduced after expert review because the differences were slight, and only observed at the lower concentration. Another laboratory study (Tavera-Mendoza et al. 2002b) did not calculate the GSI, but did report that testicular volume was reduced by 57% in *X. laevis* at NF Stage 56, after a 48-hour exposure to 21 µg atrazine/L. The SEJ for this response was reduced because of inconsistencies in the thesis upon which the publication was based, and the use of an incorrect method for measuring testicular volume, which likely resulted in this finding (see SI). Also, it is implausible that such a large tissue reduction could occur in such a brief period of exposure.

Field studies with *X. laevis* and two species of *Lithobates* were also assessed. In a field study in South Africa, no differences were observed in the GSI of male or female adult *X. laevis* collected in corn-growing regions where atrazine had been used for many years, as compared to animals from non-corn-growing regions (Hecker et al. 2004). In a Canadian field study on adult and juvenile *L. pipiens* and *L. clamitans* from agricultural and non-agricultural ponds in Southern Ontario, no differences in the GSI were reported for *L. pipiens*, and small differences were reported for *L. clamitans* (McDaniel et al. 2008). However, the authors attributed the differences to the frogs being less mature because of lower environmental temperatures, and the SEJ was reduced. *L. clamitans* were

collected during two years from several pond sites in Michigan, classified as agricultural or non-agricultural, with the atrazine concentrations measured ranging from less than 0.17 to 250 µg atrazine/L (Murphy et al. 2006b). No differences in GSI were reported for females; however, the GSI in males was significantly greater in one of the two years. The SEJ was reduced because a greater GSI was not considered to be an adverse response.

The mean score for strength was 2.29 ± SE 0.14, while that for relevance was 0.13 ± SE 0.08, and the null hypothesis was not falsified. As illustrated in Figure 11, only one study has reported evidence of a reduced GSI in fish and amphibians, and most studies produced at least moderately strong evidence (SOM > 2) of no relevant response of GSI to atrazine exposure. The evidence for no adverse effects is particularly strong for amphibians due to the greater SOM.

Cell types in gonads of fish, amphibians, and reptiles

The proportions of types of cells in the gonads are potential indicators of effects on gonadal development and function. Significant differences from normal control gonadal histology could suggest potential impacts on reproductive success, an apical endpoint. The null hypothesis for this WoE analysis was that atrazine, at concentrations commonly found in the environment, does not cause changes in numbers or proportion of cell types in the gonads. A number of studies (45 responses) have included the histological evaluation of gonadal structure, cell type, and cell stage in fish, amphibians, and reptiles. The data for the WoE assessments are shown in Figure 12.

Fish

Fewer studies have examined the gonadal cell type in fish than in amphibians. Two relatively strong studies found no differences in ovarian or testicular histology in *P. promelas* exposed for up to 30 days to 0.5 to 50 µg atrazine/L (Tillitt et al. 2010), or in a 21-day study (with levels of 25 and 250 µg atrazine/L), in which extensive male and female histological evaluations were made using a standardized protocol (USEPA 2005). A very strong study with *G. aculeatus* exposed to a range of concentrations from 0.1 to 100 µg atrazine/L, from hatching to 42 days, found no differences in the development of gonadal tissue (Le Mer et al. 2013). A 21-day study with *C. auratus* reported no histological findings in the males; however, in females, a significant increase in the proportion of atretic oocytes was reported, at exposures of 100 and 1,000 µg atrazine/L (> 100 µg/L) (Spanó et al. 2004). No significant differences in testicular cell stages or testicular pathological lesions were reported for *O. latipes* after 14 or 38 days of exposure to 0.5, 5 or 50 µg atrazine/L (Papoulias et al. 2014). This study did report greater numbers of aberrant spermatogonia, such as lagging or irregular chromosome movement, although the authors acknowledge that the relevance of these findings is unknown. In females, no pathological ovarian lesions were observed, but differences in the proportion of ovarian follicles are reported. No significant response was observed at day-14, however, at day-38, significantly fewer stage II oocytes were observed for 0.5 and 5 µg atrazine/L but not for 50 µg atrazine/L. The significance of an approximately 20% reduction in stage II oocytes at one time point is unclear. Also, the small and unequal sample sizes (3–5 fish) add uncertainty to the finding.

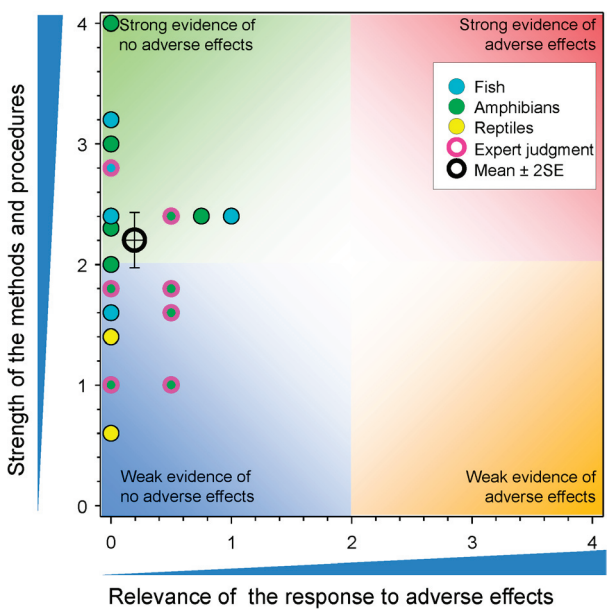

Figure 12. WoE analysis of the effects of atrazine on cell types in fish, amphibians and reptiles.

Amphibians

Studies evaluating gonadal cell type in seven species of amphibians have reported a variety of cell attributes including the number or proportion of sperm, spermatocytes, or spermatogonia, percentage of stage III testes, number of spermatogonial cell nests, frequency of testicular nurse cells, incidence of dilated tubules, and proportion of dividing gonocytes in males. The cellular characteristics reported for studies that examined ovarian tissue included the proportion of primary, secondary, or atretic oogonia, size of immature follicles, size and proportion of mature follicles, and size of the ovarian cavity. No effects were reported in laboratory or field studies with male or female *X. laevis* (Hecker et al. 2005, Smith et al. 2005), *L. pipiens* (Orton et al. 2006), *B. americanus, L. sphenochephala,* or *H. versicolor* (Storrs and Semlitsch 2008). Exposure concentrations in the laboratory studies ranged from 3 to 100 µg atrazine/L.

The field study conducted by McDaniel et al. (2008), (see section on *Concentration of steroid hormones*) reported significantly fewer juveniles with stage III testes for *L. pipiens* collected from agricultural sites, compared to those from non-agricultural sites, but no differences in adults. These authors also report significantly fewer juvenile *L. clamitans* in stage III, associated with agricultural areas (insufficient adults for comparison). As stated by the authors, differences in the maturity of frogs were observed between the sites, which likely explain these findings. Also, several other pesticides were measured in the agricultural sites, adding uncertainty to the attribution of causality. Therefore, SEJ for the data from *L. clamitans* was reduced. A laboratory study examining larval development in *L. pipiens* exposed to 15 µg atrazine/L reported no effects on the number of germ cells in the testes,

but significantly higher proportions of spermatogonia and spermatids and a lower proportion of spermatocytes, as well as a greater number of open testicular lobules (Orton et al. 2006). In females, the number of mature follicles per ovary was not different from that in control females; however, the size of immature and mature follicles was significantly larger in atrazine-exposed frogs. This study suffered from apparent husbandry issues, as demonstrated by approximately 50% of the control frogs dying, as well as other weaknesses, resulting in a low SOM score. Furthermore, intersex frogs from the controls and treatment were included in the histological evaluation, which may have confounded the interpretation. The overall evaluation of relevance for these findings was reduced (SEJ) because of serious questions about the veracity of the statistical analysis.

A study investigating sexual differentiation in *X. laevis* during metamorphosis reported decreased spermatogonial cell nests and nurse cells in the testes after a 48-hour exposure to 21 µg atrazine/L, but in a second experiment (with concentrations ranging from 2.1 to 60 µg atrazine/L), decreases in spermatogonial cell nests occurred only after 144 hours (Tavera-Mendoza et al. 2002b). The frequency of nurse cells was reduced at all concentrations after 114 hours of exposure. This study also reported a small incidence of the occurrence of Wilms'-like tumor in males. No other studies have reported similar observations in amphibians exposed to atrazine. A second paper based on this study reported findings for females (Tavera-Mendoza et al. 2002a). Differences in the proportion of primary, secondary, and atretic oogonia were reported in both the 48-hour experiment as well as the 48 to 144-hour experiment. A significant increase in the occurrence of Wilms'-like tumor was reported for the groups treated with low and high concentrations of atrazine, but not for those treated with the intermediate concentration. These studies suffered from a large number of weaknesses, some of which were major. These weaknesses included pseudo-replication, conflicting statements regarding the number of tadpoles tested, lack of consistent responses over time and across concentrations within the study, indications of poor husbandry, and inappropriate statistical analyses. The relatively low computed scores for relevance were further reduced (SEJ) due to inconsistency of responses in and between studies, "severe hemorrhaging necrosis and edema" which likely compromised findings, and temporal inconsistencies of responses (see SI for details).

Reptiles

Two species of reptiles have been examined. No effects were reported in the cell types in the Müllerian duct of *A. mississippiensis* exposed to 140, 1400, or 14,000 mg atrazine/kg (Crain et al. 1999). In a study in which eggs of *C. latirostris* were topically treated with 200 µg atrazine/L and incubated for 10 days, female hatchlings had a significantly higher proportion of type III follicles than controls (Stoker et al. 2008). The difference was small (2% controls, 5% atrazine treatment) and the route of exposure was unrealistic.

The mean score for strength was 2.20 ± SE 0.11, while that for relevance was 0.19 ± SE 0.04, and the null hypothesis was not falsified. Overall, the stronger studies (SOM > 2) indicated no effects on gonadal cell type in fish and amphibians (Figure 12). One study scored fairly high for methods (2.4) and reported effects on the *L. pipiens* (McDaniel et al. 2008); however this was a field study and causality could not be assigned to atrazine. All other responses were from weaker studies (SOM < 2) indicating weak evidence of no adverse effects.

Gonadal abnormalities of the testes

The lack of full development of the gonads or the presence of abnormalities might result in adverse effects on the apical endpoint of reproduction. The null hypothesis for this WoE analysis was that atrazine, at concentrations commonly found in the environment, does not cause abnormalities of the testes. A number of studies (22 responses, mostly in amphibians) have examined the testes for abnormalities, as they might relate to the normal development and function of the gonads. Most studies have focused on the sensitive early development when primordial gonadal tissue is differentiated into testes or ovaries. The descriptions applied to these effects in testes have included size, dysgenesis, resorption or aplasia, intersex, hermaphroditism, and "multiple" or discontinuous gonads. A lack of consistency between researchers, and in some cases, incorrect descriptive terminology, occurs in many publications. Furthermore, the use of misleading terminology such as "chemical castration" (Hayes et al. 2006a) has led to further confusion. In terms of nomenclature, we have followed the recommendations of Hecker et al. (2006). The WoE analysis for these responses is shown in Figure 13.

Fish

Four studies reported that atrazine exposure had no effect on testicular abnormalities in fish. Two were short-term studies in *P. promelas* at concentrations ranging from 0.5 to 50 µg atrazine/L (Bringolf et al. 2004, Tillitt et al. 2010). In a

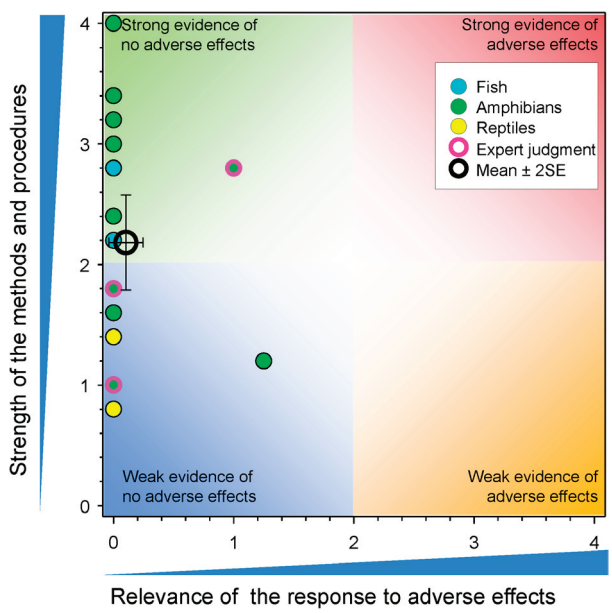

Figure 13. WoE analysis of the effects of atrazine on abnormalities in the testes in fish, amphibians and reptiles.

longer-term study, Corvi et al. (2012) reported no testicular abnormalities in *D. rerio* reared to sexual maturity (117 days) with continuous exposure to 21.6, 216, or 2,160 µg atrazine/L. Although not reported as a specific response, extensive examination and histological evaluation of the testes from a 21-day study with *P. promelas* failed to reveal obvious testicular abnormalities (USEPA 2005).

Amphibians

Ten studies have reported finding no gross or histological abnormalities in *X. laevis* (Coady et al. 2005, Jooste et al. 2005, Kloas et al. 2009a, Smith et al. 2005), *L. clamitans* (Coady et al. 2004, Murphy et al. 2006a), *L. pipiens* (Hayes et al. 2003, Orton et al. 2006), *L. tasmaniensis* (Spolyarich et al. 2010), or *H. versicolor* (LaFiandra et al. 2008). These included field and laboratory studies with exposures ranging from 0.01 to 100 µg atrazine/L.

Three studies have reported abnormalities in *X. laevis*. Hayes et al. (2002) reported up to 20% of atrazine-exposed (0.1 to 25 µg atrazine/L) frogs had "multiple gonads", but it is unclear what this means (more likely discontinuous gonads) as no data or histological evaluations were provided. Since no data or statistics were provided, a reduced SEJ was assigned. Carr et al. (2003) reported a slight but significant increase in discontinuous gonads in *X. laevis* at concentrations 25 µg atrazine/L, but not at lesser concentrations. This study suffered from husbandry issues, as demonstrated by the unusually long time required for the frogs to reach metamorphosis, and the small body size (roughly ½ of the expected weight), which could be expected to impact gonadal development. The overall evaluation of relevance was reduced (SEJ) because the increase in frequency above controls was only 4.2% and was only observed at one concentration. The increase in discontinuous gonads was later confirmed as being unrelated to sex-reversal (see section on *Sex ratios*). Another study with this species reported a failure of gonadal development, aplasia, and the occurrence of Wilms'-like tumor (Tavera-Mendoza et al. 2002b); however, as described in the section on *Gonadal abnormalities*, this study suffered from many methodological weaknesses. Furthermore, the responses reported in the testes were inconsistent with those reported elsewhere (Carr et al. 2003, Coady et al. 2005, Hayes et al. 2002). The results in these papers (Tavera-Mendoza et al. 2002a, b) and the thesis (Tavera-Mendoza 2001) are essentially uninterpretable and cannot be cited as supporting any adverse effects of atrazine on gonadal development in frogs, because of the incomplete descriptions in the methods and inconsistencies in the data. The SEJ was reduced because it is unclear how the authors determined resorption, and thus the relevance claimed is questionable. If the tissue were actually undergoing "resorption" (atrophy?), one would expect to encounter some evidence of cell loss, i.e., apoptotic or necrotic cells. In addition, the observation of failure of full development and "aplasia" (the correct term is hypoplasia) of testes also is not valid, because the gonads are still not fully developed at NF stage 56.

One study reported gonadal abnormalities in *L. pipiens* (Hayes et al. 2003). This study reported a greater incidence of gonadal dysgenesis (malformed or incompletely formed gonads) in this species after exposure to 0.1 and 25 µg atrazine/L, in the laboratory, from the age of 3 days to metamorphosis. However, no statistical analyses were performed, important information on study performance was missing, and the incidence was greater at 0.1 than at 25 µg atrazine/L. This non-monotonic response is inconsistent with all other studies, and too few concentrations were tested to properly identify such a response. A study was conducted on the occurrence of gonadal abnormalities and other reproductive biomarkers in *B. marinus*, in relation to the intensity of agricultural activity in Florida (McCoy et al. 2008). Although the number of abnormalities and frequency of intersex gonads in males increased with increasing intensity of agricultural activity, no exposures to atrazine, other pesticides, or other chemicals were measured, and the data could not be used for the WoE analysis.

Reptiles

After topical dosing (140 to 14,000 mg atrazine/kg), incubation, and hatching of the eggs of *A. mississippiensis*, no testicular abnormalities were reported (Crain et al. 1999). In a similar study, eggs of *C. latirostris* were topically dosed (200 µg atrazine/L), and no effect on testicular intratubular cell proliferation activity or apoptosis was reported (Rey et al. 2009). However, the authors did report that atrazine disrupted testicular histo-architecture, resulting in disorganized and "tortuous seminiferous tubules". This included a loss of intratubular intercellular connections and either emptied tubular lumens or luminal cellular detritus were observed. A significant increase in the seminiferous tubular perimeter and a significant reduction in the percentage of tubular perimeter occupied by cells expressing desmin were also demonstrated. These findings resulted from exposures to atrazine at concentrations greater than 100 µg/L via an unrealistic exposure route (200 µg atrazine/L in an ethanol solution).

The mean score for strength was $2.18 \pm$ SE 0.19, while that for relevance was $0.10 \pm$ SE 0.07, and the null hypothesis was not falsified. Nearly all studies demonstrated moderate to strong evidence of no adverse effects of atrazine on testicular abnormalities in multiple species (Figure 13). One amphibian study (Carr et al. 2003) with relatively strong methods reported significantly increased frequency of discontinuous testes. As stated, poor animal husbandry was demonstrated by the delayed development of the tadpoles, which confounds this finding, which is inconsistent with the findings of all other studies. Overall, there was only weak evidence of adverse effects on gonadal abnormalities.

Gonadal abnormalities of the ovaries

As in the above section on *Abnormalities of the testes*, abnormalities in the ovary might adversely affect the apical endpoint of reproduction. The null hypothesis for this WoE analysis was that atrazine, at concentrations commonly found in the environment, does not cause abnormalities of the ovary. Fewer studies (10 responses) have reported abnormalities in the ovaries than in the testes, as the primary focus of the potential effects of atrazine has been in males. The results of the WoE assessment for this response are summarized in Figure 14.

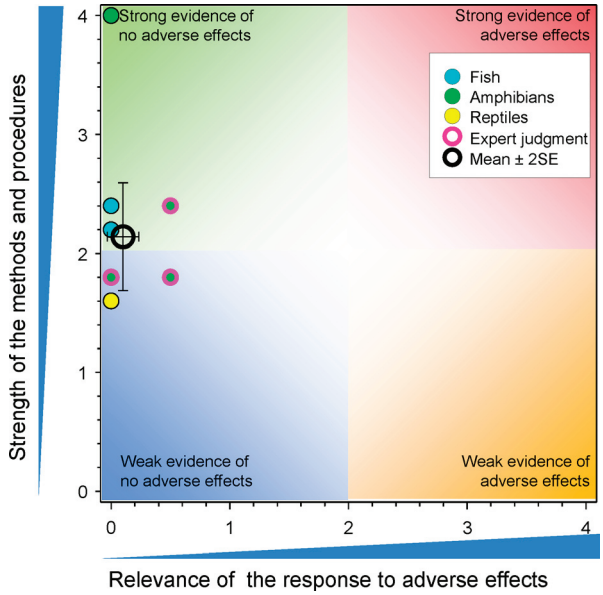

Figure 14. WoE analysis of the effects of atrazine on abnormalities of the ovaries in fish, amphibians and reptiles.

Fish

No studies have reported abnormalities of the ovary as a result of exposure to atrazine in fish. No differences in ovarian cell stages, atretic follicles, or proportion of post-ovulatory follicles were reported in *P. promelas* exposed to 25 and 250 µg atrazine/L for 21 days (USEPA 2005). Although gross ovarian abnormalities were not reported as a specific response, extensive examination and histological evaluation of the ovaries of *P. promelas* reported no gross abnormalities, which would easily have been observed. In a similar study with this species, no differences in ovarian developmental stages were observed after exposure to 5 and 50 µg atrazine/L for 21 days (Bringolf et al. 2004). Similarly, no ovarian abnormalities were observed in *P. promelas* exposed to atrazine concentrations ranging from 0.5 to 50 µg atrazine/L for 14 or 30 days (Tillitt et al. 2010).

Amphibians

A significant difference in the frequency of unpigmented ovaries was reported at all concentrations tested in one study on larvae of *X. laevis* exposed to 0.1, 0.4, 0.8, 1, and 25 µg atrazine/L (Hayes et al. 2006a). However, the statistical analysis combined pigmentation with other reported abnormalities, and it is not clear what proportion of the "malformations" were unpigmented ovaries and whether these were tested separately. Although the etiology and significance of changes in the pigmentation of the ovary is not known, the authors speculate that the increase in unpigmented ovaries was the result of androgen depletion in atrazine-treated larvae, potentially as a result of the induction of aromatase. As there was no experimental evidence provided in the study to support this theory (see section on *Expression or activity of aromatase*), and the response was non-monotonic, a reduced SEJ was assigned (see SI). An earlier study reported "multiple ovaries" in *X. laevis* at atrazine exposures of 0.1, 1.0, 10.0, and 25 µg/L (Hayes et al. 2002), but no data were provided to support this finding. The authors simply stated, "Up to 20% of the animals (16–20%) had multiple gonads (up to 6 in a single animal) or were hermaphrodites (with multiple testes and ovaries; Figure 2)." Owing to a lack of data, a reduced SEJ was assigned. Ovarian melanophores were examined by Kloas et al. (2009a). In this GLP study, repeated in two laboratories, *X. laevis* were exposed to 0.01, 0.1, 1, 25, or 100 µg/L atrazine from 8 days post-fertilization to the completion of metamorphosis. No effects on the incidence of internal melanophores were observed. Similarly, LaFiandra et al. (2008) reported no effects on ovarian development in *H. versicolor* after exposure to 20 or 200 µg/L of atrazine from Gosner stages 25 to 42, in the presence or absence of a nonlethal predator.

Another study reported changes in the proportion of primary, secondary, and atretic oogonia in ovaries of tadpoles of *X. laevis* exposed to 21 µg atrazine/L for 48 hours at NF stage 54, and in a second experiment with exposures to 2.1, 21, and 60 µg atrazine/L for durations of 48 to 144 hours at NF stage 55 (Tavera-Mendoza et al. 2002a). The responses for experiments one and two were inconsistent with respect to exposure times within the study. This publication also indicated an increase in the occurrence of Wilms'-like tumors in female tadpoles exposed to 2.1 and 60 µg atrazine/L, but not to 21 µg atrazine/L. The data for all exposure times were pooled, and the data for males and females were inappropriately combined. As previously described (see section on *Gonadal abnormalities*), these studies suffered from other weaknesses, and a reduced SEJ was assigned.

Reptiles

Only one study reported the effects of atrazine on the ovaries of reptiles. Following a single dosing of turtle eggs (*G. ouachitensis* and *G. pseudogeographica*) with 0.1, 10, or 100 µg atrazine/L, no gonadal abnormalities were observed in either species (Neuman-Lee and Janzen 2011).

While this endpoint has not been commonly included in studies on atrazine, none of the studies in fish, amphibians, or reptiles provided strong evidence of adverse effects related to ovarian abnormalities. The mean score for strength was 2.14 ± SE 0.23, while that for relevance was 0.10 ± SE 0.07, and the null hypothesis was not falsified. The strongest studies reported no effects, and the two studies in which abnormalities were reported suffered from a number of weaknesses, resulting in reduced SEJs. Overall, there was no strong evidence to link exposure to atrazine to abnormalities in the ovary of fish, amphibians, and reptiles (Figure 14).

Production of sperm and eggs

The effects on the normal production of sperm and eggs, fertility, and mating behavior, clearly have the potential to impact the apical endpoints of reproduction and the sustainability of populations of fish, amphibians, and reptiles. The null hypothesis for this WoE analysis was that atrazine, at concentrations commonly found in the environment, does not cause changes in the production of sperm and eggs. Few studies have examined this response (2 responses for sperm and 18 for eggs); the WoE analysis for production of eggs is summarized in Figure 15.

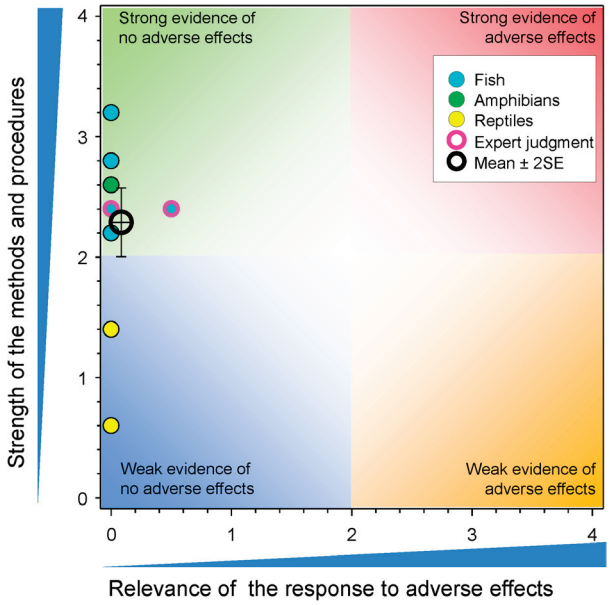

Figure 15. WoE analysis of the effects of atrazine on production of eggs in fish, amphibians and reptiles.

Fish – Sperm

Two studies have reported effects on the production of sperm in *S. salar*. Adult males were stripped of milt, exposed to 0.5, 5, 10, or 20 µg atrazine/L for 5 days, and then exposed to urine from ovulated female salmon (containing the priming hormone $PGF_{2\alpha}$) for 5 hours, to stimulate the production of sperm (Moore and Waring 1998). The authors reported a significant increase in expressible milt from fish exposed to the stimulatory urine, and a lack of significant increases in fish exposed to atrazine. However, an inappropriate statistical comparison was made. As the fish exposed to atrazine were also stimulated with urine, comparisons should have been made to the urine control rather that the negative control. In a similar study, adult males were stripped of milt, exposed to 0.5 or 5 µg atrazine/L for 5 days, and then exposed to urine from ovulated female salmon (containing $PGF_{2\alpha}$) for 5 hours, to stimulate sperm production (Moore and Lower 2001). As with the earlier study, inappropriate statistical comparisons were made. However, based on the SE bars (Figure 2 in Moore and Lower 2001), it appeared there was a significant decrease at both concentrations. As both studies were equivocal, no conclusion can be drawn, but given that the lack of effects on the GSI (see section on *Gonad-somatic index*) and the numbers and fertility of eggs (see section on *Egg hatch*), these effects are not considered biologically relevant. As only two studies were reviewed, a WoE diagram was not produced.

Fish – Eggs

Three short-term reproduction studies in *P. promelas* have reported the fecundity of females. No differences in the total number of eggs laid, number of eggs laid per day, or fertilization of eggs were reported after exposure to 25 and 250 µg atrazine/L for 21 days (USEPA 2005). In a similar study, no differences in cumulative fecundity (total number of eggs), fecundity rate (eggs/female/day), number of spawns, eggs per spawn, or fertilization rate of *P. promelas* exposed to atrazine at 5 and 50 µg/L for 21 days, were reported (Bringolf et al. 2004). In full life-cycle studies (egg to egg) of *P. promelas*, no effects on the number of spawns, eggs per spawn, total number of eggs, spawns per female or number of eggs per female were observed after 274 days of exposure to atrazine at concentrations ranging from 150 to 2,000 µg/L (Dionne 1992), or effects on spawning activity, number of eggs per spawn or eggs per female after exposure for 43 weeks to atrazine at concentrations ranging from 15 to 213 µg/L (Macek et al. 1976). Likewise, these authors observed no effects on these spawning metrics for *L. macrochirus* (18 months, exposure at concentrations ranging from 8 to 95 µg atrazine/L) or for *S. fontinalis* (306 days, exposure to concentrations ranging from 65 to 720 µg atrazine/L) (Macek et al. 1976).

A third short-term reproduction study reported significant reductions in the number of spawning events and the number of eggs produced when *P. promelas* were exposed to 0.5, 5, and 50 µg atrazine/L for 14 or 30 days (Tillitt et al. 2010). These results are not consistent with the results of the two earlier studies. The study design differed significantly from the validated regulatory guidelines used in (USEPA 2005) and (Bringolf et al. 2004) and recommended in (USEPA 2002). These differences (see SI) likely affected the results and account for the inconsistency between studies. Tillitt et al. (2010) used fewer fish per replicate, i.e., one male and two females, as opposed to the recommended two males and four females, which would have increased the variance in male performance. The data from some of the atrazine replicates where ovaries of fish were subsequently determined to contain only undifferentiated germinal cells and a few perinucleolar oocytes, should have been excluded from the analysis as they were incapable of spawning. Tillitt et al. (2010) included an analysis of mean egg production (eggs/tank with a spawning event/day) for each week, and found that there was only one week (week 3) when there was a significant difference between control and atrazine-treated groups. However, this was associated with an increase in the numbers of eggs spawned in the control group as opposed to marked reductions in the numbers of eggs spawned by atrazine-treated groups. Moreover, there were no differences among control or atrazine-treated groups in weeks 1, 2, or 4. In many respects, this calls into question the robustness of the response to atrazine. While the authors report a significant reduction in the number of spawning events in atrazine-treated groups during week 3 (treatment with 50 µg/L) or week 4 (treatments with 0.5 and 5.0 µg/L), this effect did not show time- or concentration-dependence. In the guideline study (USEPA 2005), four females and two males per tank produced approximately 300 eggs per female per week compared to 250 eggs per female per week in the study by Tillitt et al. (2010). As no data on eggs laid in the pre-exposure window were provided, the reproductive capability or state of the fish prior to initiation of the study is not known. Also, 8 of the 9 deaths recorded during the study were of female fish (Table SI 1, in Tillitt et al. 2010), suggesting possible husbandry issues. Weights measured on a subset of the males (2.8 g ± 0.3 SE) and females (1.8 g ± 0.2 SE) on day-0 of exposure were also less than those in the USEPA study (females 1.5 to 6.3 g and males 3 to 8.3 g). The SEJ for Tillitt et al. (2010) was reduced. The results reported in Tillitt et al. (2010) are inconsistent with multigenerational full

life-cycle studies with *P. promelas*, *L. macrochirus,* and *S. fontinalis*, which reported no effects on hatching success, survival, length, weight, eggs per spawn, total number of eggs, number of spawns per female, or eggs per female (Dionne 1992, Macek et al. 1976).

With a design very similar to the study by Tillitt et al. (2010) on *P. promelas*, this laboratory examined the potential effects of exposure to 0.5, 5, and 50 µg atrazine/L for either 14 or 38 days, in *O. latipes* (Papoulias et al. 2014). No significant differences were reported for spawning events; however, egg production (cumulative mean number of eggs per tank) was significantly reduced at all concentrations after 24 days, and continued until study termination. Egg production for the tanks with 14-day exposure was not significantly different between treatments. The validated OECD guideline (OECD 2012) calls for 5 females and 5 males per tank, whereas Papoulias et al. (2014) employed 4 females and only 1 male. Again, the reliance on the performance of a single male would likely have increased the variance in male performance and this may have resulted in artifactual significance in the observations. Furthermore, egg production (eggs/female/day as calculated from Papoulias et al. 2014 SI) was much smaller (4 –10 fold) than reported for this species by others (see SI), and egg mortality was much greater than expected (35% – 45% for controls and treatments; see section on *Hatching of eggs*), suggesting that poor husbandry or other factors were influential. Given these facts and a lack of concentration-response, the SEJ for this response was reduced.

Amphibians – Eggs

Egg production has been reported in two studies on *X. laevis*. In a study in which male and female *X. laevis* (F1) were exposed to 0, 1, 10, and 25 µg atrazine/L from day-4 to full sexual maturity and then mated with exposed (25 µg/L) or unexposed frogs to produce tadpoles (F2), no differences in the number of eggs per individual female frog were observed (Du Preez et al. 2008). In an *in vitro* ovulation assay, oocytes from *X. laevis* were exposed to four concentrations of atrazine ranging from 14 to 13,481 µg/L. No significant effects were observed at 135 and 13,481 µg atrazine/L, a significant increase in ovulation was observed at 1,348 µg atrazine/L, and a significant decrease was observed at 14 µg atrazine/L (Orton et al. 2009). Due to these inconsistencies of the concentration-response, a SEJ of zero was assigned.

Reptiles – Eggs

Following topical exposure to 200 µg atrazine/L and incubation of the eggs of *C. latirostris* at 30°C for 10 days, the dynamics of ovarian follicles was evaluated by quantification of the different stages of oocyte growth and maturation (Stoker et al. 2008). There were no effects of atrazine treatment on the proliferation of germ cells, as determined by the levels of incorporation of bromodeoxyuridine (BrdU). In a study on the garter snake (*Thamnophis m. marcianus*) fed 2, 10, 100, or 1,000 µg atrazine/kg for approximately 19 months, no effects were observed on the number of litters or the number of neonates produced (Walters et al. 2013). However, mortality in the control and treated animals was large.

The mean score for strength was 2.29 ± SE 0.14, while that for relevance was 0.08 ± SE 0.04, and the null hypothesis was not falsified. Overall, there is fairly strong evidence that atrazine does not affect egg production in fish, and the few studies in amphibians and reptiles present no evidence of adverse effects (Figure 15).

Testicular ovarian follicles

Testicular ovarian follicles (TOFs) are histologically defined as oocytes with an intact nucleus, nucleoli, and a surrounding squamous epithelial layer within predominantly testicular tissue (Solomon et al. 2008). The presence of TOFs may be indicative of feminization in males, and have been observed in several species of fish and amphibians as a result of exposure to 17β-estradiol (Hecker et al. 2006). This response is part of the AOP for reproduction, an apical endpoint. The null hypothesis tested in this WoE analysis was that atrazine, at concentrations commonly found in the environment, does not result in increased incidence of TOFs. TOFs have also been less accurately referred to as testicular oocytes (TOs) in some publications. Within this assessment, the term "intersex" is synonymous with the presence of one or more TOFs in a testicle. The results of the analysis of WoE for 38 responses are summarized in Figure 16.

Fish

The incidence of TOFs in *Oryzias latipes* is used frequently in assays to screen for endocrine-disrupting substances (Grim et al. 2007). The background incidence of this response in controls was characterized in this review, and TOFs were observed in control fish in 30% of all studies. The large variation between results of the four laboratories involved in the study (0 to 100% (Grim et al. 2007)) suggests that, as in frogs, the presence of TOFs in *O. latipes* is a natural phenomenon and that there may be variations between strains of fish. As for frogs (Du Preez et al. 2009), this should be considered when interpreting the incidence of TOFs in relation to exposure to atrazine (and other chemicals).

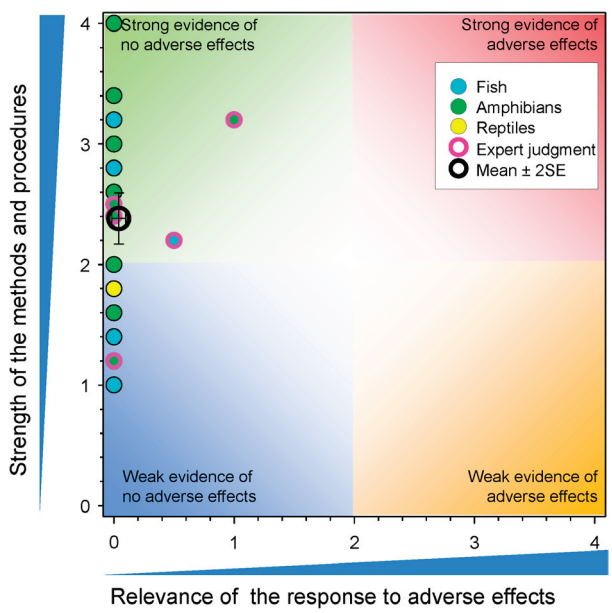

Figure 16. WoE analysis of the effects of atrazine on testicular ovarian follicles in fish, amphibians and reptiles.

No effects on the occurrence of TOFs were reported for *D. rerio* after 117 days of exposure from 17 days post-fertilization through to maturation, to 21.6, 216, or 2,160 µg atrazine/L, in duplicate experiments (Corvi et al. 2012). In a very similar study with *D. rerio,* no effect on the occurrence of TOFs was observed (Suzawa and Ingraham 2008). Likewise, no effect was observed in *P. promelas* exposed to atrazine at concentrations of 0.5, 5.0, and 50 µg/L for 14 or 30 days (Tillitt et al. 2010) or in repeated studies (2007 and 2008) in *G. aculeatus* exposed to atrazine (concentrations ranging from 0.1 to 100 µg/L) from hatching to 42 days (Le Mer et al. 2013). A field study conducted to assess the influence of WWTPs on a number of reproductive endpoints in *M. dolomieu* reported the presence of atrazine, as well as multiple other compounds, and found a high frequency of TOFs (90%–100%), but no significant differences between upstream and downstream sites (Iwanowicz et al. 2009). Because of exposures to multiple substances, causality could not be assigned.

Only one study has reported an association of atrazine with the occurrence of TOFs in fish. Smallmouth bass were sampled in 2006 and 2007 at several sites in the Potomac River watershed and at one out-of-watershed reference site (Kolpin et al. 2012). The fish were all adults of age 2 years or more, and all weighed more than 120 grams. Histological examinations of 10 and more males enumerated TOFs among other metrics. Associations between the frequency of TOFs and the contribution of WWTPs to flow, percent of land in agricultural production, number of animal-feeding operations (cattle and poultry), and number of animals in the watersheds, were examined. The study reported a correlation between concentrations of atrazine in discrete water samples and the occurrence of TOFs. A major weakness of the study was that the water samples were taken at the same time as the fish were sampled, and not when they were undergoing sexual differentiation. The authors state that, "*The purpose of sampling at SMB nesting sites was to provide chemical data to determine potential exposures to eggs and newly hatched fish, life stages shown to be most susceptible to induction of TO in other species*…"; however, they did not sample the young fish that would have been exposed to these concentrations. In fact, the authors had no measures for early life-stage exposures of the fish that they did sample. The authors acknowledged that the correlation observed in the study is not proof of causality. Also, several other significant correlations were observed between concentrations of fluoranthene, sitosterol, stigmastanol, and trans-nonachlor in the bed sediment and TOFs. The correlations between sitosterol, stigmastanol, and TOFs are mechanistically plausible as these compounds are phytosterols that may have hormonal activity. After expert review, a smaller SEJ was assigned because of the lack of appropriate measures of exposure during early development of the animals that were assessed.

Amphibians

TOFs have been reported in *X. laevis* in response to exposures to estradiol (Chang and Witschi 1956), and as occurring naturally in some species of *Rana* (Witschi 1929, 1930, 1942) and in *Acris crepitans* (Reeder et al. 2005). The number of TOFs has been reported to decrease with age in *X. laevis* (Everson 2006, Jooste et al. 2005), and regressed TOFs are more frequently observed in older animals (Jooste et al. 2005). A marked geographic variation in the incidence of TOFs in *X. laevis* has also been reported (Du Preez et al. 2009). Phylogenetic analysis of mitochondrial and nuclear genes indicated that frogs from the Southwestern (SW) Cape area of South Africa are evolutionarily divergent from those of Northeast (NE) South Africa and the rest of sub-Saharan Africa. Populations of these frogs are separated by the Cape Fold Mountains, and mature and regressed TOFs were observed in frogs from the NE sites but none were observed from the SW Cape sites. This study highlights the need for correct identification of animals used in ecological studies and the possible variation between species, strains, and populations. Furthermore, this study did not support an association between atrazine exposures and the occurrence of TOFs in *X. laevis* in South Africa, where the species is endemic.

Several laboratory studies have exposed *X. laevis* during metamorphosis and sexual differentiation to concentrations ranging from 0.01 to 100 µg atrazine/L, and reported no presence of TOFs in exposed frogs (Carr et al. 2003, Kloas et al. 2009a, Oka et al. 2008) or no significant differences in the presence of TOFs (Coady et al. 2005). It should be noted that the original designation of "intersex" by Carr et al. (2003) was based on gross observation only and that these authors report that animals classified as intersex "tended to have an obvious testicular or ovarian morphology when examined by light microscopy". A subsequent histological examination of frogs from this study by a pathologist confirmed a lack of "intersex" or TOFs (see Kloas et al. 2009a). A microcosm study was conducted, with exposures to concentrations ranging from 1 to 25 µg atrazine/L, from an age of 4 days to metamorphosis (NF Stage 66), or 10 months beyond stage 66. No differences were seen in the incidence or numbers of TOFs (stage 66) or numbers of TOFs (stage 66 + 10 months) (Jooste et al. 2005). In a companion study, juvenile frogs from the study by Jooste et al. (2005) were reared for 2 years (F1) in the laboratory with continuous exposure to atrazine, mated, and the resultant offspring (F2) were reared for 10 months (Du Preez et al. 2008). There were no differences in the number of TOFs (F1, F2) or number of TOFs per individual (F2). A field study comparing *X. laevis* caught wild in maize-growing areas with atrazine use, with those collected from non-maize areas of South Africa, reported a low incidence and no difference in the presence of TOFs (Smith et al. 2005).

No effect on TOFs in *L. clamitans* was reported at exposure to concentrations ranging from 10 to 25 µg atrazine/L (Coady et al. 2004), and a field study with this species looking at agricultural versus non-agricultural sites in Michigan reported no effect on TOFs in adults in 2 years of sampling, but a significant increase in juveniles in one of the two years (Murphy et al. 2006b). The number of TOFs per gonad was small (1 to 6), the results inconsistent between years, and as causality cannot be determined from such a field study, a reduced SEJ was assigned. A field study of frogs was conducted across wetlands in Connecticut, classified by their predominant land uses as undeveloped, suburban, urban, or agricultural (Skelly et al. 2010). While concentrations of atrazine or other compounds were not measured, of the 11 agricultural ponds, 9 had corn-cropping within close proximity. A total of 23 ponds were sampled and 233 male

L. clamitans were collected and examined for TOFs. The highest frequency of TOFs (21%) occurred in frogs from suburban land use, and both suburban and urban (16%) land uses were positively associated with the presence of TOFs. Agricultural land use (6%) was not statistically different from undeveloped sites (0%). Also, increasing proportion of agricultural land use within a pond's 200 meter buffer zone was negatively correlated with increasing frequency of TOFs. Atrazine is not used for control of residential weeds in this region (University of Massachusetts 2011), so ponds in urban areas would likely have smaller (or no) concentrations of atrazine.

No effect on TOFs in *L. pipiens* was reported in laboratory (Orton et al. 2006), microcosm (Langlois et al. 2010), or field (Hayes et al. 2003) studies. In a laboratory study also reported by Hayes et al. (2003), frogs were exposed to 0.1 or 25 μg atrazine/L throughout metamorphosis. It is stated that 29% of the animals exposed to the low concentration and 8% of those exposed to high the concentration "displayed varying degrees of sex reversal". No other data were provided. Due to the lack of statistical analysis and the inconsistency in the concentration-response, a smaller SEJ was assigned. A field study compared the incidence of TOFs in agricultural sites and non-agricultural sites in Southern Ontario and found a greater proportion of frogs with TOFs in agricultural sites (McDaniel et al. 2008). Atrazine and other pesticides were measured at the sites and the proportion of males with TOFs was correlated with the number of pesticides detected. However, as there was no significant correlation with concentrations of atrazine, a SEJ of zero was assigned.

Two publications examined the occurrence of TOFs in *A. crepitans*. Adult frogs were collected from several sites in Illinois between 1993 and 1995. No significant relationship was observed between TOFs and the detection of atrazine at the sites (Reeder et al. 1998). Also, a study examined museum specimens collected in Illinois between 1852 and 1996 (Reeder et al. 2005). There were statistically significant differences over time and among geographical regions of Illinois. The incidence declined after a high during the period between 1946 and1959, the period when atrazine was first introduced (1957), and it was found that areas with more intensive urbanization and agriculture had greater frequency of intersex. A SEJ of zero was assigned because of the lack of correlation with the introduction and use of atrazine and the failure to fulfill the Hill guideline for temporality (Hill 1965).

A laboratory study exposed the Australian frog, *L. tasmaniensis*, to 0.1, 1, 3, or 30 μg atrazine/L for 7, 14, 21, and 28 days from Gosner stages 28 to 42, and found no effect on the occurrence of TOFs (Spolyarich et al. 2010). These authors also conducted a field study in Australian rice cultivation areas with no atrazine use, and rice + corn cultivation areas with atrazine use (Spolyarich et al. 2011). Three species of *Limnodynastes* were collected between 2005 and 2007, and measured concentrations of atrazine were significantly greater in the rice + corn areas (0.70 ± 0.23 μg/L) than in the rice-only areas (0.11 ± 0.03 μg/L). The frequency of TOFs in general was low and not associated with atrazine for *L. tasmaniensis, L. fletcheri*, or *L. raniformis.* A study on the effects of mixtures of herbicides on the incidence of TOFs in *L. blairi*, from wetlands in Nebraska, reported no correlation with concentrations of atrazine (Papoulias et al. 2013). However, the frogs were exposed to a mixture of atrazine and other herbicides, and concentrations were measured at the time of collection, not during development (WoE assessment was not conducted because causality could not be assigned).

Three additional species of frogs were investigated for the presence of TOFs (Storrs-Mendez and Semlitsch 2010). This study examined gonadal morphology and the incidence of TOFs in juvenile *B. americanus, H. versicolor, and L. sphenochephala* exposed to atrazine at concentrations of 1 or 10 μg/L from Gosner stage 25 to 4 months of age. No effect of atrazine exposure on the occurrence of TOFs was reported by these authors. Similarly, in a laboratory exposure of *H. versicolor* to 0, 20 or 200 μg atrazine/L from Gosner stage 25 to 42 (including the presence or absence of a nonlethal predator), the frequency of TOFs was unaffected by atrazine (LaFiandra et al. 2008).

Reptiles

Eggs of *C. serpentina* were incubated in soil treated with the equivalent of 1.48 and 14.8 kg atrazine/ha, at a temperature of 25 ± 1°C, which normally produces only males. Histological examination of the hatching gonads provided no evidence of effects of atrazine on the occurrence of TOFs (de Solla et al. 2006). These authors suggested that small numbers of males with TOFs may be a natural phenomenon, as has been reported by other authors, and the presence of TOFs does not appear to affect fertility (Pieau et al. 1999).

The mean score for strength was $2.38 \pm$ SE 0.11, while that for relevance was $0.04 \pm$ SE 0.03, and the null hypothesis was not falsified. Across all studies for fish, amphibians, and reptiles, there was moderate to strong evidence of no effect of atrazine on the occurrence of TOFs in male animals (Figure 16). Due to the large number of studies and species, this evidence is particularly strong for amphibians. Only one laboratory study reported an association between atrazine and TOFs; however, this was a weak study (SOM = 1.2) and reported a non-monotonic response on the basis of only 2 concentrations (Hayes et al. 2003). This study is inconsistent with all other laboratory studies on amphibians. Several field studies reported the occurrence of TOFs in fish and amphibians, but variability and lack of demonstrated causality with atrazine weaken these studies.

Effects of atrazine on reproductive hormones and their actions

Concentrations of steroid hormones

There has been considerable interest in the hypothesis that atrazine is an endocrine disruptor which targets reproductive function by blocking the synthesis and actions of reproductive hormones in fish, amphibians and reptiles. This is relevant to the apical endpoint of reproduction. An evaluation of this hypothesis has focused on the direct measurement of concentrations of sex steroids, particularly 17β-estradiol and testosterone, or the enzyme aromatase which mediates the conversion of androgens to estrogens. In part, this reflects the availability

of methods for the measurement of steroid hormones and also the utility of these measures in assessing the functionality of the ovary and of the testis. The null hypothesis tested in these WoE analyses was that atrazine, at concentrations commonly found in the environment, does not result in changes in the concentrations of the steroid sex hormones.

While estradiol is often measured to assess ovarian function and vitellogenesis in egg-laying species, and testosterone and its active metabolite 11-ketotestosterone are measured in males as an index of testis function, these steroids are produced to varying degrees in both sexes and play a variety of roles including the regulation of growth, development and feedback effects within the endocrine system. Ratios of E/T were sometimes reported; however, as fish do not respond to the ratio of these concentrations, the ratios were not included in the WoE analyses. We also considered progestins, which are sex steroids produced by the ovary and testis, and play a role in oocyte development, sperm maturation, pheromonal communication and development of the reproductive tract. The section on *Secondary sexual characteristics* also deals with some of the primary actions of the sex steroids in terms of their effects on the development of secondary sex characteristics and the expression of vitellogenin, all of which are dependent on the actions of the sex steroid hormones.

Estradiol

Fish

Several studies (21 responses) have considered whether atrazine exposure affects estradiol in fish. Laboratory studies have shown that atrazine exposure has no effects on estradiol levels in the plasma of male or female fathead minnows (Tillitt et al. 2010, USEPA 2005), female *C. auratus* (Nadzialek et al. 2008) and rainbow trout (gender not clearly defined) (Salaberria et al. 2009) (Figure 17). In other studies with *C. auratus*, no changes were seen for estradiol levels in the gonads of males or females exposed to atrazine at concentrations of 100 and 1,000 µg/L for 21 days. Estradiol levels in plasma were not increased in females but were in males, but only at a concentration of 1,000 µg atrazine/L (Spanó et al. 2004). In laboratory studies with adult largemouth bass, exposure to technical atrazine had no effect on plasma estradiol levels in males, whereas estradiol levels were elevated in females exposed to 100 µg/L as a formulated product, which may have contained biologically active formulants (Wieser and Gross 2002). No effects of atrazine on whole-body concentrations of estradiol in males and females were reported in a study on *O. latipes* exposed to concentrations of up to 50 µg atrazine/L for 14 and 38 days (Papoulias et al. 2014). Finally, in a field study of modest experimental design (SOM = 1.4), Iwanowicz et al. (2009) reported no differences in plasma estradiol in female *M. dolomieu* from upstream (greater concentrations of atrazine) and downstream sites on the Conococheague Creek (Maryland U.S.). In contrast, plasma estradiol levels were reduced in males at the upstream site. A reduced SEJ was assigned as it was not possible to determine causality. The concentration of 17β estradiol in the plasma of juvenile *L. calcarifer* exposed to 0.1, 0.5, 5, 50, and 100 µg atrazine/L for 48 hours was increased with the exposure to an atrazine concentration of 5 µg/L only (Kroon et al. 2014). The SEJ was reduced because of the lack of concentration-response and the lack of internal consistency with the aromatase-response

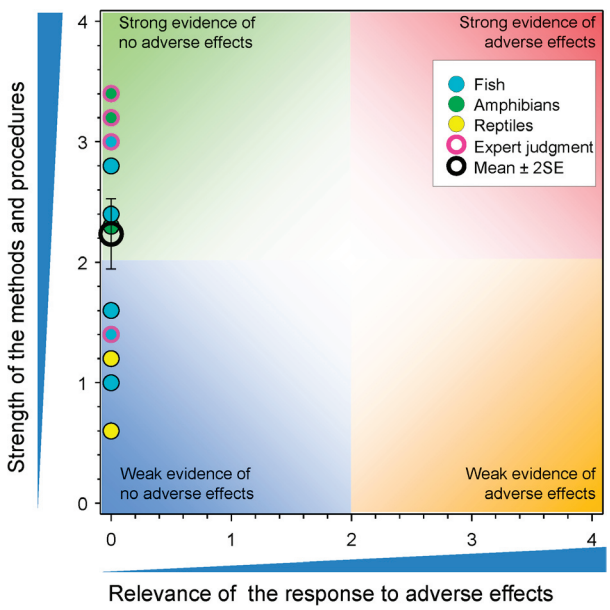

Figure 17. WoE analysis of the effects of atrazine on concentration of estradiol in fish, amphibians and reptiles.

Amphibians

Several studies (10 responses) have considered whether atrazine exposure affects estradiol in amphibians. In two years of field studies with *L. clamitans*, there were no changes in plasma estradiol levels in males or females (Murphy et al. 2006b). Similarly, McDaniel et al. (2008) found no changes in plasma estradiol in female *L. pipiens* and *L. clamitans* collected from corn-growing and non-corn-growing areas. In other studies with *X. laevis*, plasma estradiol levels were reduced in females but not in males, from corn-growing versus non-corn growing sites (Hecker et al. 2004). A reduced SEJ was assigned because the results were highly variable and were not consistent with the aromatase or GSI data. In laboratory studies with *X. laevis*, both adult males (Hecker et al. 2005) and post metamorphic females (Coady et al. 2005) showed no effects of atrazine exposure on plasma estradiol levels. Estradiol levels in postmetamorphic males exposed to atrazine were reduced (Coady et al. 2005) at a single concentration (1 µg/L) but not at concentrations of 10 and 25 µg atrazine/L. A reduced SEJ was assigned because there was a lack of a concentration-response, no effect on plasma testosterone levels, and no changes in aromatase activity. In other studies, atrazine had no effect on estradiol production by *X. laevis* ovarian fragments incubated *in vitro* (Orton et al. 2009).

Reptiles

There have been a few studies (4 responses) that have considered the effects of atrazine on estradiol levels in reptiles. Crain et al. (1997) reported no changes in estradiol levels in the chorioallantoic fluid or plasma of hatchlings of eggs of *A. mississippiensis* treated with atrazine. There were no changes in plasma estradiol concentrations in adult female *N. sipedon* (northern water snakes) exposed via diet to 2, 20, and 200 µg/L

atrazine for 3 months (Neuman-Lee et al. 2013). Moreover, the concentrations of estradiol in the serum of *C. latirostris* were not affected by topical exposure to 200 µg/L of atrazine followed by incubation of the eggs at 30°C for 10 days (Stoker et al. 2008).

Overall, there was no evidence of adverse effects on estradiol (Figure 17). The mean score for strength was 2.23 ± SE 0.14, while that for relevance was 0.00 ± SE 0.00, and the null hypothesis was clearly not falsified.

Testosterone

Fish

In laboratory studies (21 responses) with fish, there is no compelling evidence that atrazine exposure affects the production of testosterone (T). No effects were seen in the plasma of male or female fathead minnows (Tillitt et al. 2010, USEPA 2005), whole-body concentrations of estradiol in males and females of *O. latipes* exposed to concentrations of up to 50 µg atrazine/L for 14 and 38 days (Papoulias et al. 2014), female *C. auratus* (Spanó et al. 2004), largemouth bass (Wieser and Gross 2002), male Atlantic salmon (Moore and Lower 2001) and rainbow trout (gender not clearly defined) (Salaberria et al. 2009) (Figure 18). Spanó et al. (2004) reported that exposure to a large concentration of atrazine at 1,000 µg/L caused a significant decrease in plasma T after 7, 14, and 21 days in male fish. In another study, it was reported that exposure to atrazine at a concentration of 20 µg/L for 5 days appeared to increase concentrations of T in plasma, whereas the concentration of free and glucuronidated T in the bile were unaffected (Moore and Waring 1998). In a field study, Iwanowicz et al. (2009) reported no differences in plasma T in male or female smallmouth bass from upstream (greater concentration of atrazine) and downstream sites on the Conococheague Creek. The concentration of T in the plasma of juvenile *L. calcarifer* exposed to 0.1, 0.5, 5, 50, and 100 µg atrazine/L for 48 hours was increased with exposure to an atrazine concentration of 5 µg/L only (Kroon et al. 2014). The SEJ was reduced because of the lack of concentration-response and lack of internal consistency with the aromatase-response.

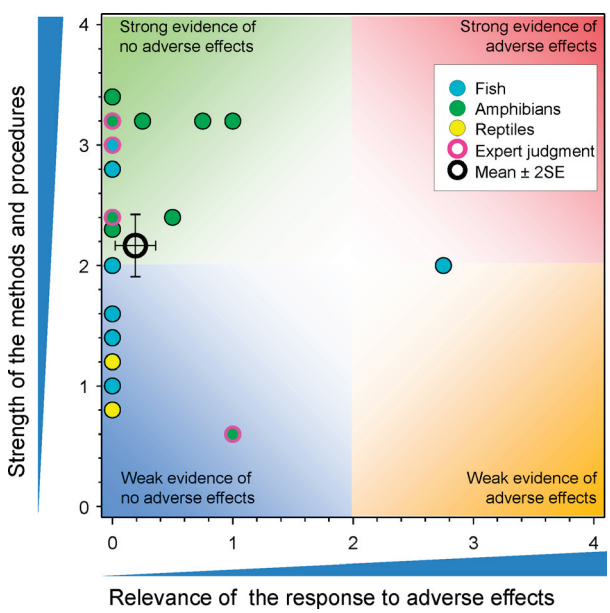

Figure 18. WoE analysis of the effects of atrazine on concentration of testosterone in fish, amphibians and reptiles.

Amphibians

The majority of studies (13 responses) have shown that atrazine exposure has no effect on T in amphibians. McDaniel et al. (2008) found no changes in plasma T in male or female *L. clamitans* or female *L. pipiens* collected from corn-growing and non-corn growing areas; however, they reported a reduction in plasma T in male *L. pipiens* from corn-growing areas during one of the three years of sampling. Murphy et al. (2006b) reported that concentrations of T in the plasma of adult female *L. clamitans* collected from agricultural sites were elevated in one of two years, but no changes were reported in the other year or in females from non-agricultural sites over two years. For males, concentrations of T were elevated in adults in one of two years, and were elevated in juveniles in one year. Hecker et al. (2004) reported lower plasma T levels in female *X. laevis* from corn-growing areas compared to non-corn growing areas, but no significant differences in plasma T levels from males at these sites. In a methodologically weak study with small sample size (SOM = 0.6), Hayes et al. (2002) reported that atrazine at 25 µg/L caused a significant reduction in plasma T levels in adult *X. laevis*. A reduced SEJ was assigned because of small sample size and the lack of measurement of aromatase in the same animals. Subsequent laboratory studies with adult males of *X. laevis* exposed to 100 µg atrazine/L (Hecker et al. 2005), and post metamorphic males and females exposed to 25 µg atrazine/L (Coady et al. 2005), showed no effects of atrazine exposure on plasma T levels. Finally, (Orton et al. 2009) reported that atrazine at the exposure concentration of 1,348 µg/L, but not smaller or greater concentrations, stimulated T production by the ovarian fragments of *X. laevis* incubated *in vitro*.

Reptiles

In studies (4 responses) with reptiles, Crain et al. (1997) reported no changes in T levels in the chorioallantoic fluid or plasma of hatchlings of eggs of *A. mississippiensis* treated with atrazine. Similarly, there were no changes in testosterone in male hatchlings of *C. latirostris* following topical treatment with 200 µg/L of atrazine at stage 20 and incubation at 33 C (male-producing temperature) for 10 days. In a study of modest experimental design (SOM = 0.6), Stoker et al. (2008) reported that treatment at 200 µg/L of atrazine followed by incubation of the eggs at 30°C for 10 days significantly reduced serum T levels in the caiman.

Overall, there was no strong evidence of adverse effects on T (Figure 18). The mean score for strength was 2.17 ± SE 0.13, while that for relevance was 0.19 ± SE 0.08, and the null hypothesis was not falsified.

11-ketotestosterone

The sex steroid 11-ketotestosterone (11-KT) is one of the prominent androgens in teleost fish, and while it is detected in many amphibian species, its physiological effects are less understood. Responses in fish and amphibians are summarized in Figure 19.

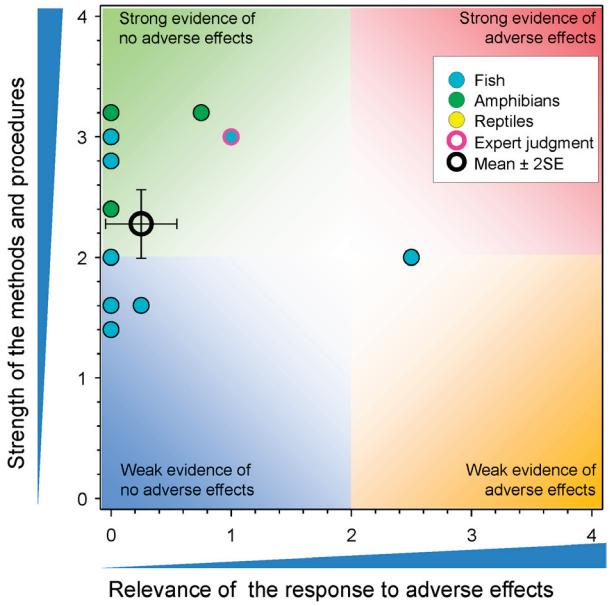

Figure 19. WoE analysis of the effects of atrazine 11-KT in fish, amphibians, and reptiles.

Fish

Studies in male (gonad and plasma) and female (plasma) of *C. auratus* exposed to 100 and 1,000 µg atrazine/L (Spanó et al. 2004), plasma of *M. salmoides* exposed to up to 100 µg atrazine/L (Wieser and Gross 2002), and plasma of male and female *P. promelas* exposed to up to 250 µg atrazine/L showed no significant effects on 11-KT. Concentrations of 11-KT in plasma of *M salmoides* exposed to atrazine at concentrations of 50 µg/L or greater, decreased by about 50% but were within the range normally reported for the species (Wieser and Gross 2002), for which reason the SEJ was reduced. In a methodologically weak study (SOM = 1.6) on *S. salar* exposed to concentrations of 0.5 to 2 µg atrazine/L, 11-KT plasma was reported to be increased by co-exposure to PGF_2 (Moore and Lower 2001); however, the experimental comparison was incorrect (see SI) and statistical significance could not be assessed (assumed to be significant). Similar experiments in the same species exposed to concentrations of atrazine from 0.5 to 20 µg atrazine/L for 5 days resulted in inconsistent changes in 11-KT in plasma, bile, glucuronidated 11-KT in bile, and release of 11-KT from testes *in vitro*. However, because incorrect comparisons were made, statistical significance could not be determined. A study of *C. auratus* exposed to 100 and 1,000 µg atrazine/L for up to 56 days (Spanó et al. 2004) resulted a decrease in 11-KT levels in the plasma of females exposed to the environmentally large exposure concentration of 1,000 µg/L for 56 days only.

Amphibians

Several studies (5 responses) reported no effects on 11-KT levels in plasma for male *L. clamitans* and male and female *L. pipiens* exposed to concentrations of atrazine from 0.006 to 250 µg/L in field studies (SOM = 3.2 to 2.4) (McDaniel et al. 2008, Murphy et al. 2006b). However, concentrations of 11-KT in adult female *L. clamitans* were elevated in one year of the two years that were studied (Murphy et al. 2006b).

Overall, the mean score for strength was 2.28 ± SE 0.14, while that for relevance was 0.25 ± SE 0.15 (Figure 19), and the null hypothesis was not falsified. This is not suggestive of biologically significant biological effects on 11-KT in fish or amphibians.

Progestins

A few studies in amphibians and fish (7 responses) have characterized the response of progestins to atrazine. Progesterone is an important precursor to androgens and estrogens in the steroid biosynthetic pathway. While progesterone alone may affect reproductive development, it is also an important precursor in fish for the synthesis of 17,20β-dihydroxy-4-pregnen-3-one (17,20βP), which mediates the final maturation of oocytes, spermiation, and, in many species, has pheromonal activity and plays a major role in preparation for spawning. The WoE analyses of the responses of progestins to exposure to atrazine are shown graphically in Figure 20.

Fish

A study in *S. salar* exposed to concentrations of 0.5, 5, 10, and 20 µg atrazine/L for 5 days measured free 17,20βP and conjugates of 17,20βP in bile, concentrations in plasma, and *in vitro* production in the testes (Moore and Waring 1998). However, incorrect comparisons were made (see SI) and statistical significance could not be determined. As a result, the SEJ was reduced for the responses in bile. However, the decrease in plasma was about 60%, and even though statistical significance was not shown, this score for relevance was not reduced.

Amphibians

Orton et al. (2009) reported increased production of progesterone in a study on fragments of the ovary of *X. laevis in vitro*,

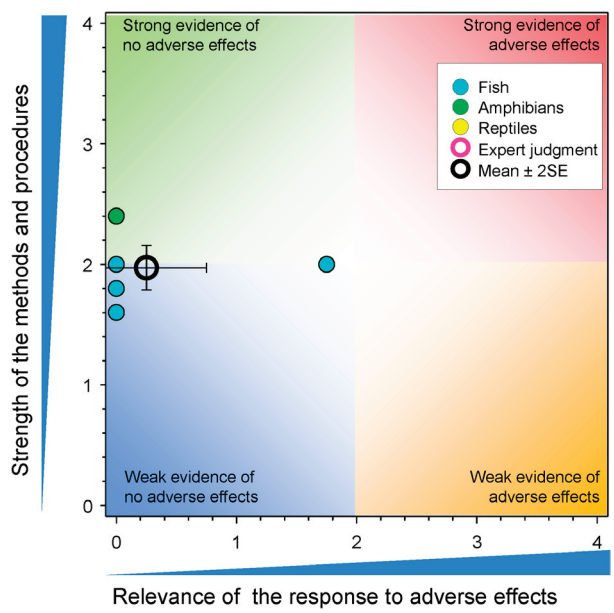

Figure 20. WoE analysis of the effects of atrazine on concentrations of progestins in fish and amphibians.

but only with exposure at concentrations of 1,348 and 13,481 µg atrazine/L (>>100 µg/L). *S. salar*, exposed to 0.5 and 2 µg atrazine/L for 5 days and then challenged with PGF2α for 5 hours, showed no significant changes in the level of 17,20βP in plasma, but the statistical comparison was incorrect (see SI) (Moore and Lower 2001). Also in *S. salar*, no effects were observed in fish exposed to atrazine at 10 and 100 µg/L in fresh water (FW) for 21 days and then transferred to salt water (SW) (Nieves-Puigdoller et al. 2007).

Overall, the mean score for strength was a moderate 1.97 ± SE 0.09, while that for relevance of effects on progestins was 0.25 ± SE 0.25 (Figure 20). The null hypothesis was not falsified, but the SE for the mean score was equal to the mean score, indicating some uncertainty. Given that progestins are precursors for androgens and estrogens, it is important to note that the observed changes in progesterone were not associated with the effects on steroids in fish and amphibians.

Expression or activity of aromatase

Much attention has been focused on the possible effects of atrazine on aromatase activity due to the pivotal role of this enzyme in mediating the conversion of C19 androgens to C18 estrogens, and ultimately, in controlling aspects of reproductive development. In part, this interest was predicated on early studies that showed that, at large concentrations, atrazine (NOEC = 65 µg/L) and several other triazine herbicides can up-regulate cytochrome P450 (CYP19) expression and aromatase activity in some, but not all, selected mammalian cells lines, when tested *in vitro* (Sanderson et al. 2000, Sanderson et al. 2001). The activity of aromatase is another line of evidence in the AOP for reproduction, and the null hypothesis for this WoE assessment was that atrazine, at concentrations commonly found in the environment, does not induce aromatase. Several studies (27 responses) have tested whether atrazine affects aromatase in fish, amphibians, and reptiles, and the WoE analysis is summarized in Figure 21.

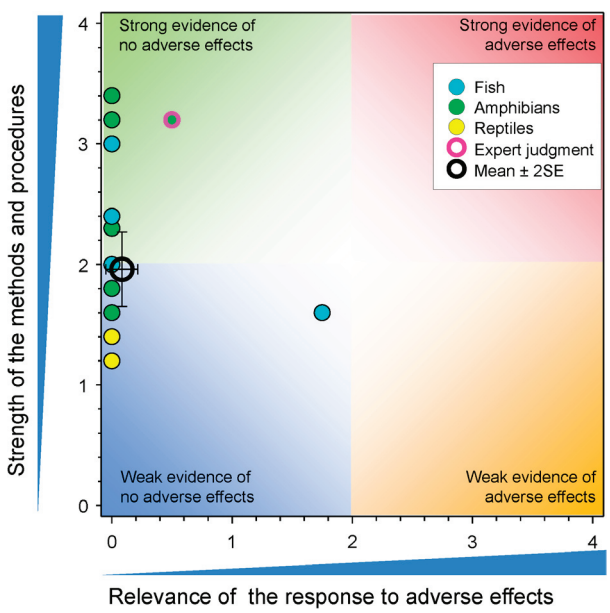

Figure 21. WoE analysis of the effects of atrazine on aromatase in fish, amphibians and reptiles.

Fish

In studies with juvenile *C. auratus* exposed to atrazine at concentrations of 100 and 1,000 µg/L for 56 days, atrazine had no effects on the expression or activity of ovarian aromatase (Nadzialek et al. 2008). Similarly, there were no effects on the activity of gonadal and brain aromatase in female *P. promelas* exposed to atrazine at concentrations of 0.5, 5.0, and 50 µg/L for 14 or 30 days (Tillitt et al. 2010). The activity of aromatase in the brain of female *O. latipes* exposed to concentrations of up to 50 µg atrazine/L for 14 and 38 days, was unaffected (Papoulias et al. 2014). Expression of RNA for CYP19B in the brain of *L. calcarifer* exposed to concentrations of 0.1, 0.5, 5, 50, and 100 µg atrazine/L for 48 hours, was unaffected (Kroon et al. 2014).

One study with juvenile *D. rerio* reported an increase in the expression of CYP19A2 after exposure to concentrations of 100 µg atrazine/L, but not at other concentrations ranging from 1 to 2160 µg/L (Kazeto et al. 2004). The SEJ for this response was reduced because of this inconsistency. No effects were reported on the expression of CYP19A1 in *D. rerio* at concentrations of 100 µg atrazine/L (Kazeto et al. 2004). In another study in juvenile *D. rerio* exposed to concentrations ranging between 21.7 and 2,170 µg atrazine/L for 3 days, increased expression of ovarian CYP19A1, but not CYP19A2, was reported (Suzawa and Ingraham 2008). Why this study was an outlier is not clear; collectively, the studies with fish do not provide evidence of strong or consistent effects of atrazine on aromatase.

Amphibians

Studies with amphibians have consistently shown that atrazine has no effects on aromatase activity or the expression of CYP19A or CYP19B in the brain or gonad of larval (Gunderson et al. 2011, Kloas et al. 2009b, Langlois et al. 2010, Oka et al. 2008), post-metamorphic (Coady et al. 2005), and adult frogs (Hecker et al. 2004, Hecker et al. 2005). Interestingly, a field study in Michigan showed that adult female *L. clamitans,* collected at agricultural sites in 2002, had significantly greater aromatase activity than frogs collected at reference sites (Murphy et al. 2006b). This effect was not observed in frogs collected in 2003. For juveniles collected in both the years, aromatase activity was elevated in the frogs from the agricultural sites. The aromatase response was not correlated with concentrations of atrazine, and the concentrations of 17β-estradiol in plasma, which would be expected to increase with greater activity of aromatase, were not elevated in the frogs from the agricultural sites (Murphy et al. 2006b). For these reasons, a reduced SEJ was assigned.

Reptiles

In studies with reptiles, the eggs of *A. mississippiensis* treated with atrazine at concentrations of up to 14,000 µg/g and then incubated at male or female-producing temperatures (Crain et al. 1997, Crain et al. 1999), showed no effect of atrazine on aromatase activity of the gonadal-mesonephros tissues or the livers of hatchlings.

The mean score for strength was 1.95 ± SE 0.15, while that for relevance was 0.08 ± SE 0.07, and the null hypoth-

esis was not falsified. The available evidence does not support an association between exposure to atrazine and altered aromatase expression or activity (Figure 21). All but one study showed no evidence of effects. One study of moderate strength (SOM = 1.6) (Suzawa and Ingraham 2008) reported the induction of expression of aromatase, but other studies of the same species failed to show a response.

Secondary sexual characteristics, including vitellogenin, in fish and amphibians

The development of secondary sexual characteristics is controlled by sex hormones released during development and/or reproductive activity. The presence of the secondary sexual characteristics is important for successful mating, and is part of the AOP for reproduction. Strictly speaking, vitellogenin (Vtg) is not a secondary sexual characteristic, but it is similarly controlled by estradiol during reproduction and was included in this WoE analysis. The null hypothesis for this WoE assessment was that atrazine, at concentrations commonly found in the environment, does not alter the expression of Vtg, laryngeal development in frogs, and other secondary sexual characteristics.

Vitellogenin

Vtg is an estrogen-dependent egg yolk-precursor protein produced in the liver of oviparous vertebrates. This protein is a hallmark of sexual maturation in females, in that the amounts increase dramatically during vitellogenesis, and this correlates with the massive increase in the size of the ovarian follicles. In contrast, this protein is poorly expressed in males, except in situations where they have been exposed to environmental estrogens. As such, the measurement of Vtg mRNA or protein has been used extensively as a marker of exposure to environmental estrogens in both males and females. Not surprisingly, several studies have considered whether atrazine exposure affects Vtg synthesis. The WoE for secondary sexual characteristics is shown in Figure 22.

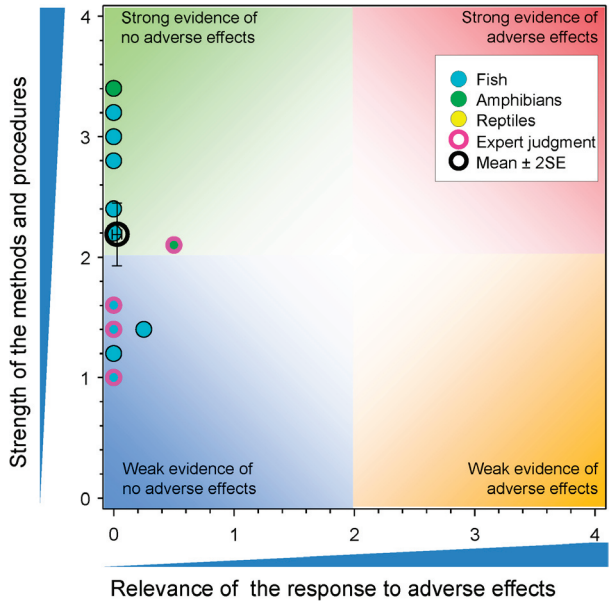

Figure 22. WoE analysis of the indirect effects of atrazine on secondary sexual characteristics in fish, amphibians and reptiles.

Fish

Many studies conducted with fish have demonstrated no association between atrazine and expression or concentrations of Vtg. For example, the expression of Vtg1 mRNA was not affected by exposure to atrazine (up to 1,620 µg/L for 120 hours) in zebrafish embryos (Muncke et al. 2007). Vtg levels were unchanged in *G. aculeatus* exposed to atrazine (up to 100 µg/L) for 42 days (Le Mer et al. 2013). Vtg levels were unaffected by atrazine exposure (up to 1,000 µg/L) in male and female adult *C. auratus* (Spanó et al. 2004), *M. salmoides* (up to 100 µg/L) (Wieser and Gross 2002), and *P. promelas* (Bringolf et al. 2004, USEPA 2005) exposed to atrazine at concentrations of up to 250 µg/L. The expression of RNA for Vtg in the liver of *L. calcarifer* was unaffected by exposure to 0.1, 0.5, 5, 50, and 100 µg atrazine/L for 48 hours (Kroon et al. 2014). The notable exception to these studies was that of (Salaberria et al. 2009), who showed that *O. mykiss* receiving injections of atrazine at 2 and 200 µg/kg body weight, experienced a small (10 µg/mL) but significant increase in plasma Vtg levels. The SEJ was reduced because the authors did not determine the sex of the fish, and as such, we cannot rule out the possibility that varying numbers of males and females or differences in the rates of maturation of the fish might have contributed to the altered Vtg levels.

In studies with *P. promelas* exposed to water collected from the Elkhorn River in Nebraska, U.S., the expression of Vtg mRNA was reduced in females and remained unaffected in males (Knight et al. 2013). The SEJ for females was reduced because a causal association with atrazine could not be demonstrated from this study. In other field studies with smallmouth bass, plasma Vtg levels were elevated in both males and females collected upstream of the Conococheague Creek (Maryland, U.S.) WWTP, where atrazine concentrations were elevated (Iwanowicz et al. 2009). The SEJ was reduced, because, again, causality could not be assigned to atrazine.

Amphibians

There have been five studies conducted which have examined the effects of atrazine on Vtg in amphibians. Of these studies, which included a range of species and developmental stages and the measurement of responses in both males and females, none showed an association between exposure to atrazine and Vtg. Atrazine had no effect on Vtg protein expression in stage-66 *X. laevis* metamorphs or in hepatocytes from adult frogs incubated *in vitro* (Oka et al. 2008). In studies with adult *L. pipiens* exposed for 7 days to water collected from the Elkhorn River in Nebraska, there was no change in hepatic expression in males or females (Knight et al. 2013). The water contained multiple herbicides (including atrazine) as well as hormones and possibly unanalyzed contaminants. The Vtg levels were measured in male *L. pipiens* and *L. clamitans* collected from areas associated with row-crop agriculture (and where atrazine and other pesticides were found) and reference areas (agricultural and non-agricultural). In these studies, there was no change in the Vtg levels between the agricultural and reference areas (McDaniel et al. 2008). While the results

of the latter two studies cannot provide evidence of causality when effects are identified, the lack of association between exposures to atrazine as a component of a mixture and adverse effects can be demonstrated.

Overall, there is no evidence to suggest that atrazine has any effects of the production of Vtg. This is consistent with the lack of effects on aromatase and estradiol discussed above.

Effects on laryngeal development in amphibians

The larynx is a sexually dimorphic organ in frogs, and it has been proposed that the size of the larynx, which is dependent on androgen levels, could be used to evaluate the response to chemicals that interfere with the synthesis or actions of androgens (see Solomon et al. 2008). In the initial study examining the effects of atrazine on the development of the larynx, Hayes et al. (2002) reported that atrazine exposure at concentrations greater than 1 μg/L reduced the cross sectional area of the larynx in *X. laevis*. The SEJ was reduced because the same response was observed in females, plus there were uncertainties around the size of the animals selected for analysis, and about the histological methods used to determine the cross-sectional area (see Solomon et al. 2008). In two additional studies, exposure to atrazine had no effect on the size of the larynx in developing tadpoles of *X. laevis* (Carr et al. 2003, Coady et al. 2005). In other studies, the laryngeal somatic index of adult female and male *X. laevis* from corn-growing (atrazine use) and non-corn-growing (no atrazine use) regions of South Africa were examined (Smith et al. 2005). These studies showed that the larynx in males was significantly larger than in the females, but there was no difference in size between frogs collected in corn- and non-corn-growing areas.

Other secondary sex characteristics

A number of other androgen-dependent secondary sex characteristics have been used in examining the possible effects of atrazine exposure in fish. Nuptial tubercles are androgen-dependent features in male fathead minnows, and effects on their development have been considered in studies of atrazine. In two studies with the fathead minnow, atrazine exposure was seen to have had no effects on the proportion of adult male fathead minnows with nuptial tubercles (Bringolf et al. 2004, Tillitt et al. 2010). Body coloration in male guppies was unaffected by exposure to atrazine (Shenoy 2012). In other studies, atrazine exposure had no effect on the production of spiggin, which is an androgen-dependent protein produced by the kidney of males of *G. aculeatus* and used in nest building (Le Mer et al. 2013). Collectively, these studies provide no evidence to support the hypothesis that atrazine directly, or indirectly, through altering endogenous androgen levels, affects secondary sex characteristics in fish.

For secondary sexual characteristics (27 responses), the mean score for strength was $2.19 \pm$ SE 0.13, while that for relevance was $0.03 \pm$ SE 0.02, and the null hypothesis was not falsified. Overall, the relevance of studies on the effects of atrazine on secondary sexual characteristics and the production of Vtg (Figure 22) confirms a lack of response across different endpoints in several species, and offers no support for indirect or direct effects of atrazine on the secondary sexual characteristics of fish or amphibians.

Studies in all-male populations of Xenopus laevis

Normal male *X. laevis* have the ZZ sex chromosomes and females have the ZW chromosomes. Two studies have reported the effects of exposure to atrazine in all male (ZZ males) groups of *X. laevis* which were produced by mating normal males with feminized males. These ZZ-females are produced by exposure of developing tadpoles to large amounts (≥ 100 μg/L) of 17β estradiol. This causes genetic males to develop into phenotypic females that are capable of reproduction when mated with normal males. Mating normal ZZ males with ZZ females results in 100% genetic males. A graphical summary of the WoE was not produced because only two studies were reported and few responses were evaluated (see SI).

In one study on ZZ males of *X. laevis* produced in this fashion and exposed to concentrations of 0.1 and 1 μg atrazine/L from 4 days post-hatch to metamorphosis, no effects on survival, sex ratio, incidence of TOFs, gonadal aromatase, or hepatic Vtg were reported (Oka et al. 2008).

In another experiment using sex-reversed parental ZZ females of *X. laevis,* the resulting progeny were exposed to a single concentration of atrazine (2.5 μg/L) (Hayes et al. 2010). Initial exposure to atrazine began "from hatching" and continued until Stage 66. No information was provided on hatching success, normal early stage mortality, or the selection of animals. Of 40 individuals (males) that were exposed to atrazine, 4 (10%) were phenotypic females based on morphology, whereas none of the controls exhibited a female morphology. Atrazine-treated males were observed to have lighter nuptial pads and smaller dermal breeding glands (based on the area of the largest gland observed in histological sections). Exposed males were also reported to have reduced concentrations of testosterone in plasma and to have a different structure of the larynx, but there were no differences in the size of the larynx between the control and exposed animals. Exposed animals had fewer testicular tubules with mature sperm bundles two years after exposure through metamorphosis, but there were no differences between exposed and control animals three years after exposure. No other differences in the testes of exposed and control animals were observed. In a mating trial (females injected with 1,000 units of human chorionic gonadotropin to induce mating), the males, which had been exposed to atrazine, were less successful in achieving amplexus, had lower concentrations of T in the plasma, and produced significantly fewer fertilized eggs.

Results observed in sex-reversed ZZ females cannot be interpreted in the context of normal frogs (*X. laevis* or otherwise). The sex-reversed ZZ females used in these studies are not found in nature. While the resulting offspring are phenotypic males, they are produced by the manipulation of sexual development of the mother by exposure to large concentrations of 17β estradiol, and nothing is known about epigenetic effects, which would be different from those in males produced in the normal manner. These data are essentially uninterpretable in the context of risk assessment and the overall evaluation of relevance (SEJ) was accordingly reduced.

Effects of atrazine on thyroid hormones

The thyroid hormones (TH: triiodothyronine, T3: tetraiodothyronine, T4) have a variety of actions in vertebrates including

effects on postembryonic growth, tissue differentiation, and reproduction (Carr and Norris 2006). Thyroid hormones play a major role in controlling metamorphosis in amphibians and smoltification in juvenile salmonids, and as such, many studies have considered the thyroid as a possible target for the effects of atrazine. The null hypothesis for this WoE assessment was that atrazine, at concentrations commonly found in the environment, does not alter concentrations of the thyroid hormones. The results of the WoE analysis are summarized in Figure 23.

Fish

A series of studies (9 responses) have evaluated the effects of atrazine on concentrations of circulating TH in *S. salar* during smoltification. In the first of these studies, salmon were exposed to atrazine at concentrations of up to 22.7 µg/L in FW for 7 days, and no effects on the concentration of T3 or T4 in plasma were reported (Waring and Moore 2004). These fish were then transferred to SW and a significant increase in the concentration of T4 was observed in the plasma of fish exposed to atrazine at 6.5 µg/L or more. Interestingly, there was no effect on the concentration of T3 in the plasma of these fish. In other studies, the concentrations of T3 or T4 in plasma were unaffected when measured in salmon smolts after they were exposed for 81 days to 0.5 and 5.0 µg/L of atrazine in FW and also 24 hours after exposure to SW. In a third study, there were no effects on plasma T3 and T4 levels in smolts in FW after a 72-hour exposure to 0.1 µg atrazine/L, or 72 hours following transfer to SW (Moore et al. 2008). In a subsequent study by other researchers, concentrations of T3 or T4 were measured in the plasma of *S. salar* parr exposed to 10 and 100 µg/L of atrazine for 21 days in FW (Nieves-Puigdoller et al. 2007). Atrazine reduced the concentration of T4 in plasma at 10 µg/L, but not at 100 µg/L. Atrazine at both concentrations did not affect the concentration of T3 in plasma. When the same fish were transferred to 30 parts per thousand (ppt) SW for 24 hours, the concentrations of T3 or T4 in plasma were reduced in fish exposed to 100 µg/L of atrazine. The concentrations of T3 or T4 in plasma were unaffected in fish exposed to the lower concentration of atrazine (Nieves-Puigdoller et al. 2007).

Overall, these studies showed that atrazine did not have a consistent effect on concentrations of TH in *S. salar* before or after transfer to SW, and when effects were seen, these were often observed at concentrations that were greater than those which would be environmentally relevant.

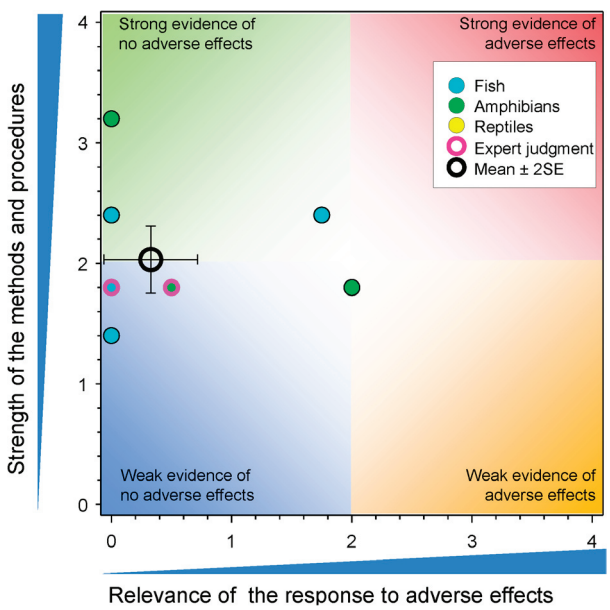

Figure 23. WoE analysis of the effects of atrazine on thyroid hormones in fish, amphibians and reptiles.

Amphibians

Only a few studies (3 responses) have considered thyroid hormones in amphibians, and in doing so, a variety of endpoints and species were examined. The concentrations of T4 were measured in the plasma of larvae of *A. tigrinum* exposed to atrazine at 75 and 250 µg/L for 86 days (Larson et al. 1998). There were no changes in the concentration of T4 in salamanders at stage 2 (early in the metamorphic climax). However, at stage 4 (middle of the metamorphic climax), there was a significant elevation in the concentration of T4 in the plasma of larvae exposed to 75 and 250 µg/L of atrazine. The SEJ was reduced because of small effects and inconsistency across stages. In *X. laevis* exposed to 1, 25, and 100 µg atrazine/L from 8 days post-fertilization to metamorphosis, there was no effect on thyroid follicular cell hypertrophy, hyperplasia, and colloid content (Wolf 2012). Other studies showed that the expression of deiodinase 3 (dio3) in the tail of tadpoles of *L. pipiens* was reduced, following exposure to atrazine at measured concentrations of 3.7 µg/L, from Gosner stages 27 to 42, metamorphic climax (Langlois et al. 2010). This result was not consistent with the results of other studies, which have shown that atrazine exposure does not affect metamorphosis (see section on *Successful development…*).

A single study considered the effects of atrazine on thyroid hormones in reptiles. In this case, eggs of the snapping turtle were exposed to atrazine at 1.48 and 14.8 kg/ha via treated soil, at male-producing temperatures (de Solla et al. 2006). A histological examination of the thyroid gland revealed no effect on thyroid morphology.

The mean score for strength was 2.03 ± SE 0.14, while that for relevance was 0.33 ± SE 0.19, and the null hypothesis was not falsified. Overall, there was no strong evidence to suggest that environmentally relevant concentrations of atrazine have adverse effects on thyroid hormones.

Direct effects of atrazine on cortisol and stress physiology

A number of studies (16 responses) have been conducted to characterize the stress response of fish and amphibians following exposure to atrazine (Figure 24). Most studies have measured cortisol as a biomarker of stress. The corticosteroid response is an adaptive response to a variety of stressors and facilitates a response that enables an organism to respond to that stressor. As such, it is not evident that mounting a corticosteroid response is adverse, or would contribute to reductions in fitness on its own, thus making the attribution of relevance more challenging. The null hypothesis for this WoE assessment was that atrazine, at concentrations commonly

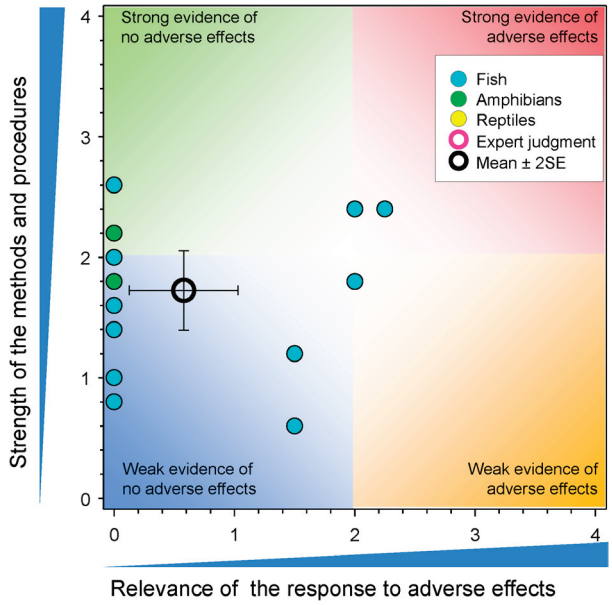

Figure 24. WoE analysis of the effects of atrazine on stress physiology in fish, amphibians and reptiles.

found in the environment, does not alter the concentrations of cortisol.

Fish

Several studies that reported the effects of atrazine on plasma cortisol levels in fish used unrealistic exposure concentrations, and SEJs of zero were assigned. For example, studies with *O. mykiss* (Shelley et al. 2012b), and *R. quelen* (Cericato et al. 2008, Cericato et al. 2009), showed no effects at atrazine concentrations of up to 5,100 µg/L. In *C. carpio* (Gluth and Hanke 1985), and *S. salar* (Nieves-Puigdoller et al. 2007), exposures to atrazine concentrations of approximately 100 µg/L led to elevated levels of cortisol. Other studies have shown that atrazine had no effect on cortisol levels in fish. For example, the cortisol content in larvae of *F. herteroclitus* was unaffected following exposure to three salinities (3, 15, and 35 ppt) and atrazine concentrations of 5, 50, and 500 µg/L for 24 hours (Fortin et al. 2008). Similarly, in studies with adrenocortical cells from *O. mykiss*, atrazine at concentrations ranging from 0.01 to 100,000 µg/L had no effect on dibutyryl cAMP-stimulated cortisol production (Bisson and Hontela 2002). However, in the same study, exposure to atrazine at concentrations ranging from 108 to 1,080 µg/L (> 100 µg/L) caused a statistically significant decrease in cortisol production, while much higher concentrations (108,000 µg/L) caused an increase in the production of cortisol. It was also unexpected that all exposure concentrations caused the same decrease (25%) in the production of cortisol. The concentration of cortisol increased in the plasma of *R. quelen* exposed to formulated atrazine + simazine at a large concentration of 1,740 µg/L for 96 hours, followed by a 45, 90, 135, and 180-day "recuperation" phase without stress test (Koakoski et al. 2014). When fish were stressed (i.e., chased with a net for 60 sec) the concentration of cortisol deceased.

In contrast to the studies listed above, others have reported that exposures to atrazine increased cortisol levels in fish. The freshwater fish *P. lineatus* had elevated levels of plasma cortisol at 1 and 3 hours after exposure to atrazine at concentrations of 10 µg/L (Nascimento et al. 2012). In studies with *S. salar*, exposure to atrazine at concentrations of 6.5 µg/L or greater in FW, led to elevated plasma cortisol levels when measured in FW, and also 24 hours after transfer to SW (Waring and Moore 2004). In other studies with *S. salar*, Nieves-Puigdoller et al. (2007) showed that exposure to atrazine at a concentration of 100 µg/L, but not 10 µg/L, led to elevated cortisol levels in fish held in FW and also for 24 hours following transfer to SW.

Amphibians

Few studies (3 responses) have considered the effects of atrazine on corticosteroids in amphibians. In the larvae of the tiger salamander, *A. tigrinum*, exposure to atrazine at concentrations of 75 and 250 µg/L for 86 days had no effect on plasma corticosterone levels (Larson et al. 1998). *In vitro* studies examining adrenal and kidney cells of *X. laevis* stimulated by the adrenocorticotropic hormone (ACTH) showed that exposure to atrazine at concentrations ranging from 2.16 to 21,600 µg/L had no effect on the production of corticosterone (Goulet and Hontela 2003). In other studies with the adrenal and kidney cells of the bullfrog, exposure to atrazine at concentrations of 2,160 µg/L or more, significantly decreased ACTH- and dbcAMP-evoked corticosterone secretion (Goulet and Hontela 2003).

One study on the effects of mixtures of pesticides that contained atrazine reported effects on cortisol (Hayes et al. 2006b). Because the effect of atrazine alone was not characterized, it was not possible to assign causality. In addition, because of a weakness in the experimental design, it was not possible to determine if the observed effects were the result of concentration-addition, response-addition, synergism, antagonism, or a combination of all of these (LeBlanc and Wang 2006). This study was not included in the WoE.

In many respects, these results are consistent with what has been reported in mammals, where recent studies have demonstrated that atrazine, at much greater exposures than those tested in aquatic organisms, and several of its metabolites, activate the hypothalamic-pituitary-adrenal (HPA) axis in rodents, leading to increases in the circulating concentrations of ACTH and corticosterone (Foradori et al. 2011, Fraites et al. 2009, Laws et al. 2009, Modic 2004, Pruett et al. 2003, Pruett et al. 2009, Schwab et al. 2005). Overall, the mean score for strength was 1.73 ± SE 0.17, while that for relevance was 0.58 ± SE 0.23, and the null hypothesis was not falsified. It should be noted that the corticosteroid response is generally adaptive. In the absence of relevant effects on apical endpoints (discussed above), short-term changes in cortisol most likely are adaptive and do not have negative biological consequences.

Effects of atrazine on the smoltification of fish

Effects on the physiology of smoltification

Smoltification is a key developmental event in the life history of juvenile salmonids. This involves the development of tolerance to SW, which results from a reorganization of the major osmoregulatory organs including the gill, gut, and kidney, and is accompanied by an increase in activity of Na^+/

K^+-ATPase at the gills and the ability to regulate plasma ions following transfer to SW. Not surprisingly, when examining the effects of atrazine on smoltification, many studies have also considered endpoints that relate to the endocrinal, physiological, and the ion-osmotic responses that are associated with the changes in osmoregulatory balance that accompany the smoltification process. These responses are summarized in Figure 25. The null hypothesis tested in this WoE assessment was that atrazine, at concentrations commonly found in the environment, does not alter the physiology of smoltification in fish.

The results of studies (31 responses) examining the effects of atrazine on smoltification have been highly variable. In general, these studies have also shown that atrazine does not affect the physiological responses that are thought to mediate the smoltification process. For example, in the studies by Moore et al. (2003), exposure to atrazine did not affect the activity of Na^+K^+ ATPase, or concentration of Cl^- and Na^+ in plasma. In other studies, (Moore et al. 2007) reported an inconsistent effect on the activity of gill Na^+K^+ ATPase, in that the activity during the freshwater phase was reduced in fish exposed to atrazine at a concentration of 1 µg/L but not at 5 µg/L. Upon transfer to SW, there was no difference in the activity of Na^+K^+ ATPase in relation to atrazine exposure. Studies by Matsumoto et al. (2010) reported no changes in the activity of Na^+ K^+ ATPase in the gills, plasma ions (Na^+, K^+, Mg^+ or Cl^-) and water content of the muscles in *S. salar* parr, following exposure to atrazine at concentrations of 10 or 100 µg/L for 21 days in FW. In other studies, Nieves-Puigdoller et al. (2007) reported no changes in the activity of Na^+ K^+ ATPase in the gills of *S. salar* parr following exposure to atrazine at 10 or 100 µg/L for 21 days in FW. However, upon transfer to SW, the activity of gill Na^+, K^+ ATPase was reduced in those fish previously exposed to atrazine at 100 µg/L. In the same studies, atrazine had different effects on plasma ions. Atrazine at 100 µg/L increased the concentrations of sodium, chloride, calcium, and magnesium in plasma, whereas atrazine at 10 µg/L only increased the concentrations of calcium in plasma (Nieves-Puigdoller et al. 2007).

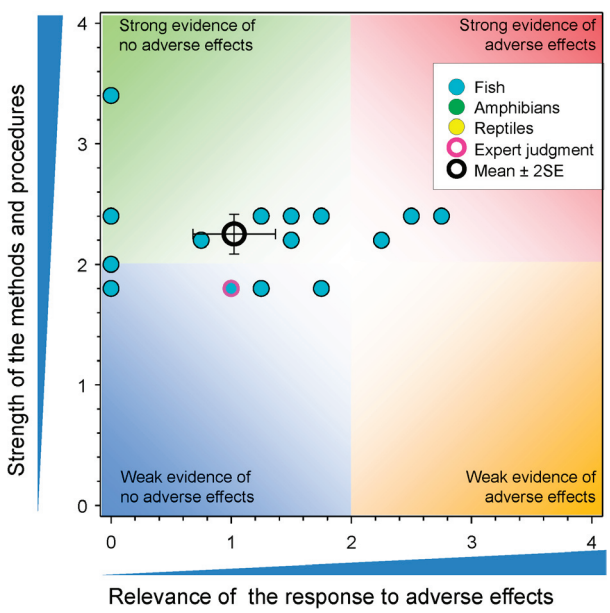

Figure 25. WoE analysis of the effects of atrazine on physiological responses related to smoltification in anadromous fish.

In studies on fish exposed to atrazine and then transferred to SW (see section on *Smoltification of fish and survival of salt-water challenge*), a variety of physiological responses indicative of altered smoltification were observed (Waring and Moore 2004). These included a significant decrease in the activity of Na^+K^+ ATPase in the gills of fish exposed to atrazine at concentrations of 2 µg/L or more for 5 days, followed by challenge to SW, increases in plasma Na^+, Cl^-, K^+ and plasma osmolality in fish exposed to atrazine in FW and after transfer to SW. Interestingly, there was no effect on the water content of muscle.

Several of these studies (Moore et al. 2008, Nieves-Puigdoller et al. 2007, Waring and Moore 2004) have also reported the levels of hormones that are thought to be integral to the smoltification process. In general, these studies provided limited evidence that hormonal levels were targets for the actions of atrazine. These are discussed in the section on *Effects on thyroid hormones*.

The above data for physiological responses associated with smoltification showed a mean score for strength of 2.25 ± SE 0.08, while that for relevance was 1.02 ± SE 0.17 (Figure 25). The null hypothesis was falsified, suggesting that exposures to atrazine may affect physiological responses related to smoltification.

Smoltification of fish and survival of salt-water challenge

Survival in SW is generally used as the ultimate measure of whether salmonid fishes have successfully completed smoltification. This is an apical endpoint, and here the null hypothesis was that atrazine, at concentrations commonly found in the environment, does not alter or decrease survival of anadromous fish when they are transferred to SW. Several studies have used this measure to assess the effects of atrazine on smoltification, and have come to varied conclusions. For example, some of these studies have shown that *S. salar* can survive a SW challenge following exposure to atrazine at up to 2 µg/L for 7 days (Moore et al. 2003), 5.0 µg/L for 81 days (Moore et al. 2007), 100 µg/L for 21 days (Nieves-Puigdoller et al. 2007), and 100 µg/L for 4 days (Matsumoto et al. 2010). However, other studies have shown that exposure to atrazine negatively affects survival following transfer to SW. In studies by (Moore et al. 2008), all fish exposed to a relatively low concentration of atrazine (0.1 µg/L) survived in FW, but there was 100% mortality 72 hours after transfer to SW. Other studies showed that exposure to atrazine at concentrations of 13.9 µg/L or greater, for 7 days, resulted in 28% mortality following SW transfer (Waring and Moore 2004). In a separate study by Waring and Moore (2004), exposure of *S. salar* to 5 µg/L or more of atrazine for 7 days resulted in mortality of up to 43% following transfer to SW.

Collectively, these studies provide some evidence of the effects of atrazine on smoltification (Figure 26). The mean score for strength was 2.14 ± SE 0.24, while that for relevance was 1.11 ± SE 0.53, and the null hypothesis was falsified. However, the results reported in the literature are dichotomous, some showing effects and others not. At this time, it is not clear whether the differences in responses to atrazine

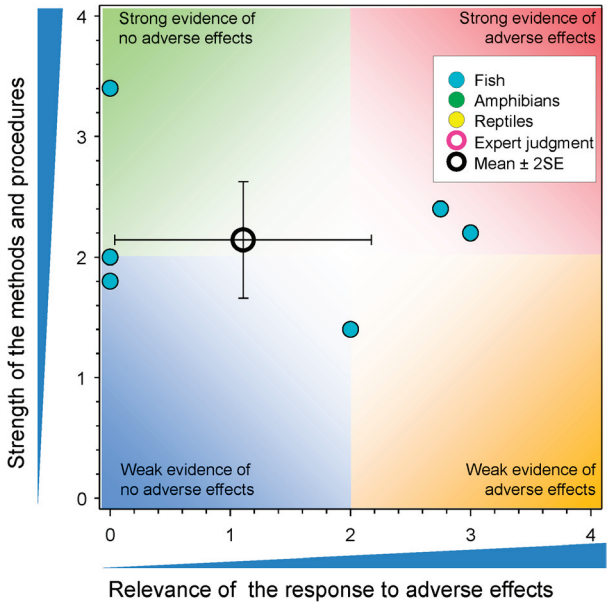

Figure 26. WoE analysis of the effects of atrazine on survival of salt-water challenge in anadromous fish.

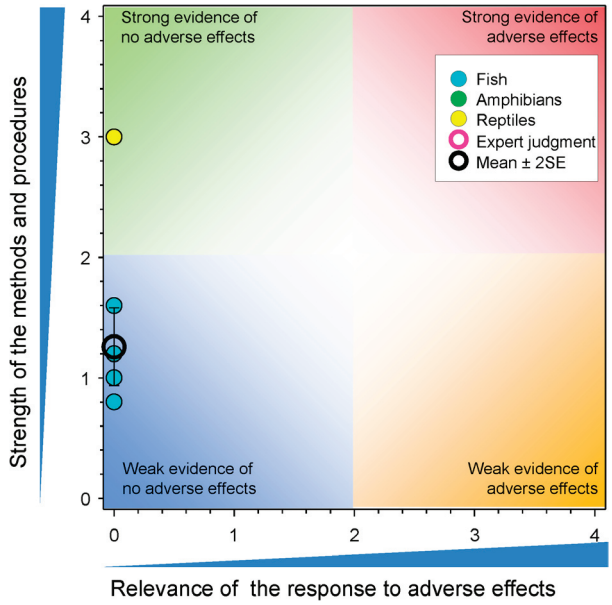

Figure 27. WoE analysis of the effects of atrazine on physiological and biochemical processes *in vitro*.

are due to differences between populations of *S. salar* in the Eastern and Western Atlantic, or to differences in techniques between laboratories.

Effects of atrazine on physiological and biochemical responses

A large number of studies have reported the effects of atrazine on biochemical and physiological responses *in vitro* and *in vivo*. These are discussed in the following two sections.

Physiological and biochemical effects in vitro

A number of studies (14 responses) have reported effects on various physiological and biochemical processes of cells and tissues of fish and reptiles *in vitro*. Without exception, these responses were only observed at large concentrations of atrazine (>3,000 µg/L), and a SEJ of zero was assigned to all studies (Figure 27). The null hypothesis for this WoE assessment was that atrazine, at concentrations commonly found in the environment, does not alter physiology and biochemistry in the cells and tissues of fish and amphibians *in vitro*.

Exposure to atrazine concentrations greater than 10,000 µg/L caused no response in the activity of glutathione-s transferase from the liver of *O. kisutch* (Trute et al. 2007). *In vitro* studies on a cell line (ZC-7901, derived from the epidermis of the snout of *Ctenopharyngodon idellus*) showed no effects on concentrations of intracellular Ca^{++}, chromosome breakage, reactive oxygen species, mitochondrial potential, ATP depletion, apoptosis, DNA ladder formation, or cytotoxicity, at atrazine concentrations of 3,000 µg/L or greater in the growth medium (Liu et al. 2006). In a study on human-hERα and the *A. mississippiensis*-aERα, concentrations of atrazine between 0.2 µg/L and 21,500 µg/L failed to induce any competitive binding with estradiol, or even a concentration-response (Rider et al. 2010). In an *in vitro* study using *Micropogonias undulatus* (Atlantic croaker), effects of atrazine on progestin-induction of oocyte maturation in the ovarian tissue, and binding to the progestin receptor on the ovarian membrane, were reported at large concentrations (≥2,160 µg atrazine/L) (Thomas and Sweatman 2008). Overall, there were no relevant effects for these endpoints (Figure 27) The mean score for strength was 1.26 ± SE 0.16, while that for relevance was 0.00, and the null hypothesis was clearly not falsified.

Physiological and biochemical effects in vivo

A large number of studies (262 responses) have reported the effects of atrazine on physiological and biochemical endpoints in fish and amphibians *in vivo* (Figure 28). No responses in reptiles were found in the literature. Considering the large number of responses assessed and the wide range of effects

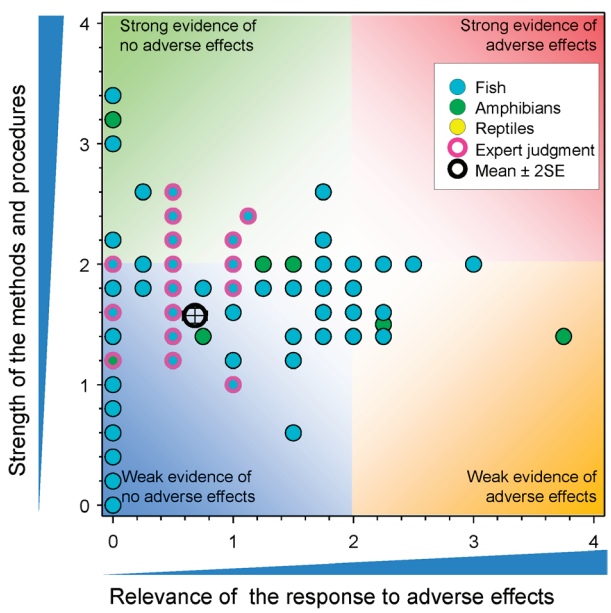

Figure 28. WoE analysis of the effects of atrazine on physiological responses in fish and amphibians.

reported, the discussion of these in the following sections has been grouped by species.

Fish

A number of studies reported no effects of atrazine on the physiology of fish. A study in *C. auratus* exposed to atrazine concentrations of 5, 10, or 15 µg/L for up to 6 days showed no effects on micronucleus formation (Cavas 2011). No effects were observed on a number of enzymes and other physiological responses, including degradative enzymes, in *C. carpio* exposed to atrazine via the diet, to atrazine concentrations of up to 1,000 mg/kg diet for 84 days (Cossarini-Dunier et al. 1988). In other studies on the same species, no effects on the expression of mRNA or the concentration of the enzyme inducible nitric oxide synthase (iNOS) in the brains of *C. carpio* exposed to atrazine at concentrations less than 428 µg/L for 40 days (Wang et al. 2013). A similar lack of effect was reported for the content of malondialdehyde (MDA) in the gills and brain and the activity of catalase (CAT) in the brain and kidney of the same species (Xing et al. 2012a, Xing et al. 2012c). No effects on the ultrastructure of renal cells were reported in the Brazilian fish *Caquetaia kraussii* and *Colossoma macropomum* exposed to atrazine at concentrations of 2500 µg/L for 72 hours (de Bravo et al. 2005). Similarly, no effects were reported in the expression of ovarian mRNA encoding antioxidant proteins or the content of malondialdehyde in the livers of *D. rerio* exposed to atrazine concentrations of up to 1,000 µg/L for 14 days (Jin et al. 2010b). No effects on the content of protein, lipid, and water, or residual mass were reported in larval *F. heteroclitus* exposed to up to 500 µg atrazine/L for 96 hours (Fortin et al. 2008). No effects were reported on the activity of acetylcholinesterase in the brain of *L. macrochirus* exposed to atrazine concentrations of up to 1,000 µg/L, for up to 96 hours (Mehler et al. 2008). No effects on the content of mRNA in the brain were observed in *M. salmoides* 96-hours after being dosed with atrazine at a concentration of up to 3 µg/g (Martyniuk et al. 2009). Several studies on *O. mykiss* have reported no effects on a number of endpoints: the expression of glutathione-S-transferase in the liver (up to 200 µg atrazine/L for 6 days (Salaberria et al. 2009)), olfactory responses (up to 100 µg atrazine/L for 10 minutes (Tierney et al. 2007)), liver-somatic index, protein in blood, and hematocrit (up to 555 µg atrazine/L for 96 hours (Shelley et al. 2012b)). No effects were reported on the basal sodium concentration in blood or the basal activity of Na^+K^+ ATPase in the gills of *P. lineatus* exposed to atrazine at a concentration of 10 µg/L for 24 hours (Nascimento et al. 2012). Similarly, Paulino et al. (2012b) observed no effects on the basal activity of Na^+K^+ ATPase and carbonic anhydrase (CA) (up to 25 µg atrazine/L for 4 and 14 days) or the abundance of mitochondria-rich cells (MRCs) in the gills of *P. lineatus* exposed to atrazine at concentrations of up to 25 µg/L for 14 days (Paulino et al. 2012b). In a related study on the same species of fish, no effects were reported on the activity of superoxide dismutase (SOD), CAT, glutathione peroxidase (GPx), and glutathione-S-transferase (GST), or glutathione (GSH) content, lipid peroxidation, or morphology of the gills (Paulino et al. 2012a). In *P. promelas* exposed to atrazine concentrations of up to 1,000 µg/L for up to 96 hours, no effects were reported on the activity of acetylcholine esterase (AChE) in the brain (Mehler et al. 2008). In the same species, exposure for 7 days to river water containing atrazine had no effects on the hepato-somatic index or expression of ERα in males (Knight et al. 2013). No effects were seen on the histology of the gills, activity of CA in the gills, activity of several enzymes in the liver, and AChE, in the Brazilian fish *R. quelen* exposed to atrazine at concentrations of up to 100 µg/L. In *S. ocellatus*, no effects on degradation of protein (exposure to concentrations up to 80 µg atrazine/L for 8 days (McCarthy and Fuiman 2008)) or respiration (up to 80 µg atrazine/L for an unspecified time (del Carmen Alvarez and Fuiman 2005)), were reported. As discussed in the section on smoltification, in studies on *S. salar* exposed to up to 100 µg atrazine/L for 14 days, no effects were observed on the activity of AChE in the brain, the hepato-somatic index, or the activity of Na^+K^+ ATPase in the gills (Nieves-Puigdoller et al. 2007). In the same species exposed to up to 100 µg atrazine/L and then challenged with SW, no effects were reported on the concentration of Na^+ in plasma, content of water in muscle, and activity of Na^+K^+ ATPase in gills (Matsumoto et al. 2010).

A number of other studies reported physiological and biochemical effects in fish after exposure to atrazine. A study on *C. auratus* reported that exposure to formulated atrazine at concentrations of up to 15 µg atrazine/L for up to 6 days, resulted in increased incidence of four genotoxicity endpoints (Cavas 2011). A reduced SEJ was assigned because the formulated product was used, and it was not possible to assign causality to atrazine or other constituents of the formulation. A weak study (SOM = 0.8) reported effects on various hematological and serum components and enzymes in *C. auratus* and *Oreochromis niloticus* exposed to large concentrations of 3,000 and 6,000 µg atrazine/L for up to 28 days (Hussein et al. 1996).

Five studies have reported effects on biochemical and physiological endpoints in *C. carpio*. The effects on glucose in blood, cytosolic lactate, activity of glucose-6-phopsphatase, glycogen content in muscle, and glycogen reserves in the liver, were reported in fish exposed to a diet containing atrazine at levels of 10, 100, and 1,000 mg/kg for 14 and 84 days (Cossarini-Dunier et al. 1988); however, the SEJ for these was reduced because of the inconsistency or lack of concentration-response. In the same study, the glycogen content in the liver, liver-somatic index, 5-nucleotidase, Na^+K^+ ATPase, and AChE activity in muscle were affected only at the large exposure concentration of 1,000 µg atrazine/L. The concentration of sialic acid in mucus was significantly decreased by 40% to 50% in fish exposed to atrazine at levels of 10, 100, and 1,000 mg/kg for 14 and 84 days (results reported but data not shown). A concentration-response was not observed, there was no effect apparent on immune challenge or on survival, and the authors did not link these responses to apical endpoints. Also, in carp, Fu et al. (2013) reported induction of CYP 4501A, B, and C mRNA, as well as increased amounts of and activity of CYP 450 in fish exposed to atrazine at concentrations up to 428 µg/L for 40 days. A reduced SEJ was assigned because the response is adaptive, recovery was observed, and atrazine had no apparent effect on survival, even at exposures greater than 100 µg/L. A weak study (SOM = 0.6) in the same species reported effects on cholesterol, protein, and glucose in serum, as well as glyco-

gen in the liver and muscle of fish exposed to atrazine at a concentration of 100 µg/L for 6, 24, and 72 hours (Gluth and Hanke 1985). Survival was not reported, but these responses are also adaptive and probably of inconsequential relevance. Wang et al. (2011), also working with *C. carpio*, reported effects on the expression of IL-1β, IL-1R1, and IFN-γ2b mRNA in the spleen and head kidney, but the authors did not link these to apical endpoints. The activity of GPx in the gill and kidney of *C. carpio* only increased after a 40-day exposure to atrazine at 42.8 µg/L, but not to atrazine at 4.28 and 428 µg/L (Xing et al. 2012a, Xing et al. 2012c), but the relevance of this response was reduced by SEJ because it was judged to be adaptive. Similarly, decreases in the activity of AChE and expression of its mRNA in the brain and muscle of the same fish were reported (Xing et al. 2010a, Xing et al. 2010b), but the scores for relevance were reduced by SEJ because of inconsistency in the concentration-response and the lack of an explainable mechanism (atrazine is not an inhibitor of AChE). A number of other effects were reported in the same fish upon exposure to atrazine at 4.28, 42.8, and 428 µg/L for 40 days. These were: CAT activity in the liver and gills (Xing et al. 2012b), and carboxylesterase in brain (Xing et al. 2010a) and muscle (Xing et al. 2010a). Why atrazine would inhibit carboxylesterases is unclear, it contains no carboxy-ester moieties and would not be expected to interact with the active site of this enzyme. The activity of GPx in the liver and brain (Xing et al. 2012a, Xing et al. 2012b), GST in the liver, brain, kidney, and gill, as well as differences in the kappa, theta, mu, and rho isoforms of GST in the liver, kidney, brain, and gill (Xing et al. 2012c), were also altered. Differences were also observed in the histopathology of the liver, gill, brain, and kidney (Xing et al. 2012a, Xing et al. 2012b), the activity of iNOS in the brain (Wang et al. 2013), and MDA content in liver (Xing et al. 2012b) and kidney (Xing et al. 2012a). Differences in the activity of superoxide dismutase (SOD) in liver, brain, gills, and kidney (Xing et al. 2012a, Xing et al. 2012b),were also reported between control and fish exposed to atrazine at a concentration of 42.8 µg/L or more. As with many similar observations discussed in this section, these are most likely biomarkers of exposures and have not been linked to apical endpoints. A number of blood parameters (cell numbers, hemoglobin, protein, glucose, protein, ions, and activity of AST, ALT, ALP, LDH) were affected in *C. carpio*, when exposed to very large concentrations of atrazine (5,000 to 30,000 µg/L) for 96 hours (Blahová et al. 2014). In contrast, the red blood cell count, albumin, and concentrations of neopterin were unaffected.

All of the studies reported by Xing et al., (discussed above) were apparently conducted on the same experimental fish (e.g., the weights were all the same) but only one of the papers (Xing et al. 2012b) reported an abnormal response on behavior and feeding. The authors reported that fish exposed to atrazine at 428 µg/L showed abnormal behavior, including decreased intake of food, slower swimming speed, and unresponsiveness to outside stimuli. No weights were reported and no statistical tests were conducted, but this was assumed to be significant; however, this suggests that overt toxic responses were caused at the exposure. Why this was not reported in the other papers (discussed above) or how this affected the other responses reported by the authors is unclear.

Two studies in *C. punctatus* exposed to atrazine for 1 to 35 days at extremely large concentrations ranging from 4,238 to 10,600 µg/L (and as a commercial formulation) reported increases in micronuclei, damage to DNA in erythrocytes, and damage to the gills, but no mortality (Nwani et al. 2010, Nwani et al. 2011).

Twelve studies in *D. rerio* reported a range of physiological and biochemical responses. A relatively strong study (SOM = 2.8) reported increases in the activities of GPx and glutathione reductase (GR) at atrazine concentrations of up to 30 µg/L, and decreases at atrazine concentrations of 90 µg/L. The activity of GST decreased at a concentration of 90 µg atrazine/L and the activity of catalase decreased at all concentrations (Blahová et al. 2013). Thiobarbituric acid-reactive substance (TBARS) increased at atrazine concentrations of 30 and 90 µg/L. The increases in activity at greater concentrations of atrazine were likely because of the induction of metabolic pathways and were not considered adverse; a reduced SEJ was assigned. Survival was unaffected, but there was a 17% reduction in the rate of growth at 90 µg/L (Blahová et al. 2013), suggesting that relevance to apical endpoints was moderate, but only at the greatest concentration tested. In a study on the behavior of *D. rerio,* it was reported that the activity of AChE in brain, but not in muscle, was decreased after exposure to a large atrazine concentration of 1,000 µg/L for 14 days (Schmidel et al. 2014). The effect was not observed at the atrazine concentration of 10 µg/L. Also in the same species, Dong et al. (2009) reported increased activity of aminopyrine N-demethylase (APND), NADPH-P450 reductase, erythromycin N-demethylase (ERND), and NADPH-P450 reductase, and the content of cytochrome P450 in liver in fish exposed to atrazine at concentrations ranging from 10 to 1,000 µg/L for 5 to 25 days. A reduced SEJ was assigned because of inconsistent concentration-response and the fact that most of these responses were adaptive. At atrazine exposures from 0.1 to 12.5 µg/L for 3 days, the expression of mRNA related to immune function and production of reactive oxygen species (ROS) and nitrous oxide (NO), but not in other genes, was reported (Jin et al. 2010a). All responses were observed only at the greatest tested concentration. At atrazine exposures of 10, 100, and 1,000 µg/L for 14 days, the activity of antioxidant enzymes and the content of glutathione increased in the liver (Jin et al. 2010b), but in the ovary, increases were observed only at an atrazine concentration of 10 µg/L and the GSH content in the ovary was reduced at 1, 100, and 1,000 µg atrazine/L. The content of MDA in the liver increased with exposure to atrazine at concentrations greater than 100 µg/L, but not in the ovary. The expression of mRNA associated with antioxidant protein increased at atrazine concentrations of 10 and 100 µg/L in liver but there were no effects in the ovary at atrazine concentrations up to 1,000 µg/L. The mRNA associated with the expression of genes of the inner membrane of the mitochondria was increased at atrazine concentrations of 10 µg/L and 100 µg/L, but not at 1,000 µg/L(Jin et al. 2010b). Exposure to atrazine at concentrations of 10 and 1,000 µg/L for 14 days caused non-significant, histopathological changes in the liver (a concentration-response was observed), as well as changes in the content of some proteins in the liver (Jin et al. 2012). Increased occurrence of lesions in the liver was

observed but not quantified in zebrafish exposed to atrazine at 90 µg/L for 28 days (Plhalova et al. 2012). This did not affect survival, but the rate of growth was slightly reduced (17%). The induction of soluble and microsomal GST was reported in eggs of *D. rerio* exposed from 2 to 48 hours post-fertilization to an atrazine concentration of 5,000 µg/L (Wiegand et al. 2000). In another study, microsomal GST was induced at atrazine concentrations of 1,000 µg/L or greater, in fish exposed from 2 to 48 hours, but soluble GST was induced at atrazine concentrations ranging from 100 to 10,000 µg/L. Survival was unaffected; the 48-hour LC50 was 36,800 µg atrazine/L (Wiegand et al. 2000). Detoxification of atrazine was observed in the same studies. Changes in the expression of genes and proteins were reported in *D. rerio* exposed to atrazine at 0.3, 3, and 30 µg/L from 1 to 120 hours post-fertilization (Weber et al. 2013); however, the relationship to growth or other apical endpoints was not characterized. Damage to DNA and changes in the activity of three enzymes associated with the detoxification of xenobiotics were reported in zebrafish exposed to large concentrations of atrazine ranging from 2,500 to 10,000 µg/L for 21 days (Zhu et al. 2011). At lower concentrations (10, 100, and 1,000 µg atrazine/L for up to 25 days), the same group reported changes in the activity of microsomal GST (Zhu et al. 2010), but because of inconsistencies in response over time, reduced SEJs were assigned. However, damage to DNA was reported at all concentrations tested (Zhu et al. 2010). Overall, most of the responses associated with exposure of zebrafish to atrazine at realistic concentrations were associated with adaptive responses, and apical endpoints, where reported, were affected only at concentrations greater than 100 µg/L. Given the lack of effect on growth and survival at atrazine concentrations up to 2,160 µg/L in a strong (SOM = 2.8) study on *D. rerio* (Corvi et al. 2012), the relevance of all these adaptive responses to apical endpoints is questionable.

The rate of gill ventilation in juvenile *L. calcarifer* was unaffected by exposure to atrazine at concentrations of 0.1, 0.5, 5, 50, and 100 µg/L for 48 hours (Kroon et al. 2014). Exposure of larvae of *F. heteroclitus* to 5 µg atrazine/L, but not to 50 and 500 µg atrazine/L, for 96 hours at two salinity levels (15 and 35 Practical Salinity Units (PSU)), resulted in a small increase in dehydration but not on the water content of the body (Fortin et al. 2008). A small increase in hyperhydration was reported at 5 µg atrazine/L and 3 PSU only. SEJs of zero were assigned because of weaknesses in the statistical analysis, the small differences in the proportion of dehydrated and hyperhydrated larvae relative to the controls, the large 95% CIs, and the lack of effect on water content.

Studies on *Gobiacypris rarus*, the Chinese rare minnow, exposed to atrazine concentrations ranging from 3 to 333 µg/L for 28 days, reported effects on the histopathology of the gill and kidney (Yang et al. 2010b). No significant effects were observed on the expression of mRNA for Na^+K^+ ATPase, glucocorticoid receptor (gr), heat shock protein 70 (hsp70), and heat shock protein 90 (hsp90) in the gill, but there was a concentration-response for Na^+K^+ ATPase. The same genes were unaffected in the kidney of males but were affected by atrazine exposure in females. This latter response was inconsistent between sexes in this species, and there was no consistent concentration-response. For these reasons, a reduced SEJ was assigned. In a parallel study in the same species exposed to the same concentrations, the hepato-somatic index and the size of hepatocytes was increased in males and females, and expression of the androgen receptor (*ar*) was increased by approximately two-fold in males exposed to atrazine concentrations of 100 µg/L or more (Yang et al. 2010a). Expression of the estrogen receptor (*er)* and androgen receptor (*ar)* in the liver of females was increased by about five-fold at atrazine concentrations of 33 µg/L or more. A reduced SEJ was not assigned, but the design of the study was pseudoreplicated and may be unreliable. The expression of mRNA for hsp70 was increased in the liver of male and female *G. rarus.* A non-monotonic, inverted U-shaped concentration-response was observed for hsp70, but was not characterized by the authors. A U-shaped, non-monotonic concentration-response (not characterized) was also observed for mRNA of hsp90, but both of these observations may be unreliable because of pseudo-replication of fish in a single tank per treatment.

Changes in 28 proteins in the liver of *M. salmoides* were observed 96 hours after an intraperitoneal injection of a large dose of 3,000 µg atrazine/kg (Sanchez et al. 2009). Following injection with a smaller dose (3 µg atrazine/kg), the expression of four unidentified genes were changed after 96 hours (Sanchez et al. 2011). The responses to other contaminants were larger; fish treated with cadmium, PCB-126, phenanthrene, and toxaphene had 126, 118, 137, and 58 genes, respectively, which were differentially expressed. Relevance to survival was not reported, but presumably was not significant.

A number of studies have reported the effects of atrazine on physiology and biochemistry of *O. mykiss.* Renal histology was affected by exposure only to exposure concentrations of 1,400 and 2,800 µg/L for 96 hours (Fischer-Scherl et al. 1991). Unquantified histological changes also were reported after exposure to nominal atrazine concentrations of 5, 10, 20, 40, and 80 µg/L for 28 days, but significance could not be determined (Fischer-Scherl et al. 1991). Cytological alterations in proximal and distal nephronic tubules following exposure to atrazine at 10, 20, 40, 80, or 160 µg/L for 45 days were reported but not analyzed statistically, so no significance could be assigned (Oulmi et al. 1995). Expression of the gene for CYP1A in liver was decreased after exposure to atrazine at 2 or 200 µg/L for 6 days, and the activity of catalase was increased only at a concentration of 200 µg/L (> 100 µg/L) (Salaberria et al. 2009). The differential expression of 653 genes was reported and the spleen-somatic index was affected only after exposures to 555 µg atrazine/L for 4 days (Shelley et al. 2012b).

The exposure of *P. lineatus* to air stress (removal of the fish from water for 3 minutes) after a 24-hour exposure to 10 µg atrazine/L resulted in changes in the number of red blood cells, basal hematocrit, and hemoglobin (co-variants), basal glycogen in the liver, and basal glucose in the blood (Nascimento et al. 2012). These responses were all likely secondary to the effects on cortisol, and are considered part of a normal stress response (see section on *Direct effects of atrazine on cortisol*). In the same species of fish exposed to atrazine concentrations of 2, 10, and 25 µg/L for 48 hours and 14 days, Paulino et al. (2012a, 2012b) reported effects on gill morphology, mucus cell types in the gill, the activity of CA in the gill, activity

of SOD, CAT and GST, and lipid peroxidation after 14 days, but only at 10 μg atrazine/L, not at 2 or 25 μg/L, and the SEJs were reduced because of inconsistency in the concentration-responses. These responses are all probably related and are a general response to stress.

The exposure of female (but not male) *P. promelas* for 7 days to a pulse of water containing atrazine in a mixture of contaminants, increased the hepato-somatic index by 55%, and decreased the expression of ERα (Knight et al. 2013), but because of the presence of other potential and unidentified stressors, the SEJ was assigned a value of zero.

The histology and activity of several enzymes (decreases in CAT, GR, and GST) in the liver of *R. quelen* were affected after 96-hour exposures to atrazine at concentrations of 2, 10 and 100 μg/L (Mela et al. 2013). The effects on concentrations of Cl^- and Mg^{++} in plasma and the activity of renal CA were also observed, but the differences were small and the concentration-responses were inconsistent, resulting in a reduced SEJ.

The exposure of *S. ocellatus* to atrazine at concentrations of 40 and 80 μg/L resulted in a significant increase in the rate of protein synthesis (McCarthy and Fuiman 2008), but responses were inconsistent over time and lacked a concentration-response relationship, resulting in a reduced SEJ.

Several studies have reported physiological responses in *S. salar* in relation to smoltification (see *Effects on physiology of smoltification*). The physiological responses are also summarized here in the context of physiology. Studies on Na^+K^+ ATPase in the gills of *S. salar* exposed to atrazine at concentrations of 0.5 and 5 μg/L for 81 days in FW reported decreased activity, but no difference after transfer to seawater (SW), and no effect on survival (Moore et al. 2007). This resulted in the assignment of a reduced SEJ. In a later study on the same species exposed to 0.1 μg atrazine/L for 72 hours in FW and then transferred to SW, reductions in activity of the same enzyme were observed in FW and SW (Moore et al. 2008). All exposed fish died when transferred to SW, in contrast to the observations in the 2007 study in which fish were exposed to greater concentrations. A number of responses were reported in *S. salar* exposed to 10 and 100 μg atrazine/L in FW for 21 days (Nieves-Puigdoller et al. 2007). Feeding was reduced at 100 μg/L, HSI was increased in females only and only at 100 μg/L, glucose in plasma was increased but only at 100 μg/L, and Na^+ increased at 10 μg/L and decreased at 100 μg/L, along with Cl^-, Ca^{++}, and Mg^{++}. The effects in salmon discussed above were from studies of moderate strength (SOM = 1.8–1.4) and are in contrast to the lack of responses in a stronger study (SOM = 3.4) for similar endpoints at similar concentrations (Matsumoto et al. 2010).

A very weak study (SOM = 0) on *S. mossambicus* exposed to formulated atrazine at a large concentration of 1100 μg/L for up to 90 days, reported effects on osmoregulatory ions and whole-body water (Prasad and Reddy 1994). The authors also reported that exposures to 1100 μg formulated atrazine/L for 7 to 35 days resulted in various effects on muscle and liver (Prasad and Reddy 1994).

Amphibians

A number of studies have reported no effects of atrazine on physiological and biochemical endpoints in a range of species of amphibians. A study on genotoxic responses in tadpoles of *B. americanus* exposed to atrazine concentrations ranging from 250 to 10,000 μg/L for 21 days reported no effects (Freeman and Rayburn 2004). In *L. pipiens*, no effects were observed on a number of biomarkers in animals exposed to 2.1 μg atrazine/L for 21 days and then challenged with the pathogenic fungus, *Bd* (Paetow et al. 2012). No change in expression of 5β-reductase in the liver was observed in *L. pipiens* exposed to atrazine at 3.7 μg/L in microcosms for 100 days (Langlois et al. 2010). In the same species exposed for 7 days to a pulse of river water containing atrazine (time weighted average (TWA) concentration of 4.8 μg/L) mixed with other stressors such as herbicides and hormones, no effects were observed on the liver-somatic index in males and females and on the expression of *erα* in females (Knight et al. 2013). No effects on hematocrit were observed in *L. pipiens* exposed to atrazine at concentrations of 20 and 200 μg/L, from Gosner Stage 25 to metamorphosis (Allran and Karasov 2000). A number of studies on *X. laevis* also reported no effects. No effects were reported in a weak study (SOM = 0.6) on the histology of liver and fat-body or total ADP/ATP in *X. laevis* exposed to 400 μg atrazine/L from NF stage 47 to 58 or 62 (Zaya et al. 2011a, Zaya et al. 2011b). No effects on markers of genotoxicity were reported in *X. laevis* exposed to atrazine at concentrations ranging from 200 to 800 μg/L for 1–3 weeks (Freeman and Rayburn 2004).

Some studies have reported physiological and biochemical effects in frogs exposed to atrazine. Expression of *erα* in the brain was significantly increased in *L. pipiens* exposed to atrazine at a concentration of 3.7 μg/L for 100 days in microcosms (Langlois et al. 2010). The same response was observed in female *L. pipiens* exposed for 7 days to river water containing atrazine (TWA of 4.8 μg/L) mixed with other herbicides and hormones (Knight et al. 2013); however, as causality could not be assigned, a SEJ of zero was assigned. There was no effect on gene expression of the nuclear receptor steroidogenic factor-1 in the brains of tadpoles of *L. catesbeianus* exposed to atrazine at concentrations of 1 and 100 μg/L for 48 hours (Gunderson et al. 2011). In adults of the same species, breathing rate was affected after exposure to atrazine concentrations ranging from 560 to 20,000 μg/L (> 100 μg/L) (Allran and Karasov 2001).

Genotoxic responses to atrazine were reported in other experiments on *X. laevis* at various developmental stages when exposed to atrazine concentrations over the range of 200 to 800 μg/L for 1 to 5 weeks (Freeman and Rayburn 2005). Atrazine is not classified as genotoxic (USEPA 2006) and no plausible mechanism has been suggested in this and in an earlier study (Freeman and Rayburn 2004) that would account for the difference in uptake of dyes by the nuclei, although this may be related to general toxicity on exposure to these large concentrations. Apoptosis was increased in the developing brain and kidney of larvae of *X. laevis* exposed to very large concentrations (25,000 and 35,000 μg atrazine/L) for 48 hours (Lenkowski et al. 2008). Immunocytochemical activity of caspase-3 in tadpoles of *X. laevis* at NF stage 62 was increased

following exposure to a large exposure concentration of 400 µg atrazine/L from NF stages 47 to 62 (Zaya et al. 2011a). The same authors (Zaya et al. 2011b) also reported changes in the expression of genes (acyl-CoA dehydrogenase, glucocorticoid receptor, and peroxisome proliferator-activated receptor-β) in *X. laevis* exposed to a large atrazine concentration of 400 µg/L for 24, 48, and 72 hours or 14 days. Langerveld et al. (2009) reported reduced weight of the fat body and changes in the expression of genes in *X. laevis* exposed to atrazine at a large concentration 400 µg/L.

In a study on several biomarkers in *L. catesbeianus* larvae at stage-28, exposed to atrazine at concentrations of 5, 10, and 20 µg/L for 7 days, no effects were reported on the content of cholesterol and protein in gills, and triglycerides in muscle (Dornelles and Oliveira 2013). However, effects were reported for cholesterol in liver and muscle; glycogen in the gills, liver, and muscle; lipid peroxidation in liver, gills, and muscle; total lipids in liver, gills, and muscle; total protein in liver and muscle; and triglycerides in gills and liver (Dornelles and Oliveira 2013). These responses were likely biomarkers of exposure or normal compensatory responses to a xenobiotic.

A significant loss of mass due to dehydration at reduced humidity was reported in *A. barbouri* exposed to atrazine at concentrations of 4, 40, or 400 µg/L from Harrison-stage 28 to metamorphosis and then observed on day-137 and day-239 after the last exposure (Rohr and Palmer 2005). However, observed losses in mass were in the range of 10%, and their significance (and linkage) so long after removal of atrazine is doubtful. A study on the endosymbiotic algae in the eggs of *A. maculatum* suggested that these algae were lost as a result of exposure to concentrations of 50 to 400 µg atrazine/L (Olivier and Moon 2010). This observation had a large score for relevance, but these algae have been isolated from eggs and tested for sensitivity to atrazine (Baxter et al. 2014). The 96-h EC50 for growth from four pooled experiments was 299 µg atrazine/L (± SE of 16.3) and the EC10 was 147 ± 22.5 µg/L, suggesting *de minimis* risks to this species via inhibition of the alga.

The mean score for strength of the large number of studies on physiological effects was 1.57 ± SE 0.03. The score for relevance was 0.57 ± SE 0.05 (Figure 28), and the null hypothesis was not falsified. However, the relevance of almost all of the physiological and biochemical responses to the apical endpoints of survival, growth, development, or reproduction, as discussed above, were not established. Most of these responses were likely adaptive and were related to normal metabolism of or response to exposure to xenobiotics. In most cases, they did not result in changes in survival, even at large exposure concentrations (> 100 µg/L). Given the lack of large or significant responses in terms of survival and growth of fish, amphibians, and reptiles observed upon exposure to atrazine (see other response in sections above), these effects do not appear to be relevant to apical endpoints.

Effects on immune function

Immune function in fish, amphibians, and reptiles is not as well understood as it is in mammals, but a number of studies have reported the effects of atrazine on various immune endpoints in fish and amphibians. The results of the WoE assessments for these responses (53) are summarized in Figure 29.

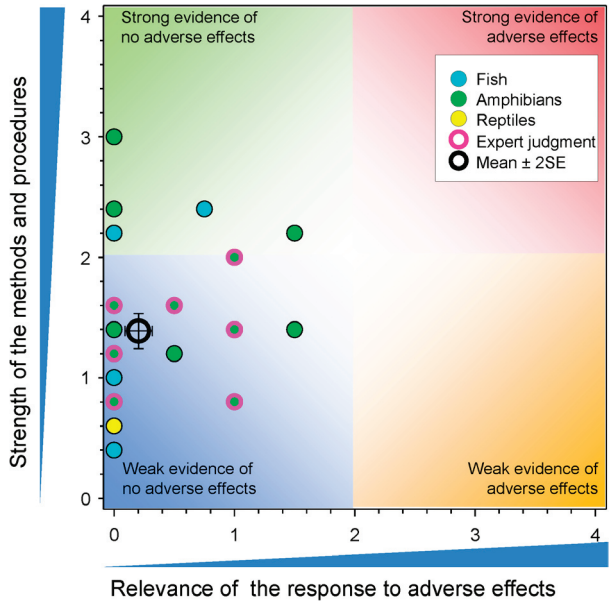

Figure 29. WoE analysis of the effects of atrazine on immune function in fish and amphibians.

The null hypothesis for this WoE assessment was that atrazine, at concentrations commonly found in the environment, does not alter immune function.

Fish

Seven studies in fish reported no effects on several immune endpoints. No effects of atrazine exposure were reported on phagocytosis of *Saccharomyces cerevisiae* and *Yersinia ruckeri* vaccine by macrophages of *C. carpio* exposed to atrazine *in vitro* at concentrations of 1,750 to 28,000 µg/L for 2 hours (Cossarini-Dunier 1987). Similarly, the titer of spring carp virus (SCV) in a cell line of *C. carpio*, exposed to 28,000 µg atrazine/L *in vitro* was unaffected after a 3-day exposure (Cossarini-Dunier and Hattenberger 1988). In *C. carpio* fed a diet containing 10 to 1,000 mg atrazine/kg (equivalent to daily doses of 100, 1,000, and 10,000 µg/kg b.w./d) for 84 days, there was no effect on the production of antibodies to *Yersinia ruckeri*, weight of the spleen, or number of lymphocytes in the spleen and kidney (Cossarini-Dunier et al. 1988). No effects on lysozymal activity in the plasma of juvenile *O. mykiss* was observed following exposure to 59 or 555 µg/L of atrazine for 4 days (Shelley et al. 2012b).

Others have reported effects of atrazine on immune function in fish. In one study, six cytokinins involved in innate immunity were measured in *D. rerio* following exposure to atrazine at concentrations ranging from 0.1 to 12.5 µg/L for 3 days. Of these, the expression of one cytokine, IL-1β, was increased, but only at 12.5 µg/L (Jin et al. 2010a). Following exposure of juvenile *O. mykiss* to up to 555 µg/L of atrazine for 4 days, the proportion of lymphocytes in blood decreased and monocytes increased, but only at a large concentration of 555 µg/L (Shelley et al. 2012b). In a study on the same species, the viability of unstimulated and LPS-stimulated peripheral blood leukocytes was reduced only at the atrazine concentration of 2,160 µg/L (Shelley et al. 2012a). The proliferative index of peripheral blood leukocytes was decreased after

in vitro exposure to atrazine at 216 and 2,160 µg/L for 96 hours (Shelley et al. 2012a). The number of intraceolomic cells and the phagocytic index of these cells in *R. quelen* exposed to formulated atrazine at a large exposure concentration of 1,020 µg/L for 24 hours was decreased (Kreutz et al. 2010). Also, in *R. quelen* exposed to 1,020 µg atrazine/L (> 100 µg/L) for 24 hours and 10 days, phagocytic index, serum peroxidase, bactericidal activity, bacterial agglutination, and lysozymal activity were altered; however, complement natural hemolytic activity was not (Kreutz et al. 2012, 2013).

Amphibians

Several studies have reported no effects on immune responses in amphibians. No effects of formulated or technical atrazine were observed on the production of protein and antimicrobial activity of skin secretions in metamorphs of *X. laevis* exposed to 0.1 to 1,000 µg/L from NF stages 49–50 to 66 (Gibble and Baer 2011). In a weak study (SOM = 1.4) on *L. pipiens* exposed to 1 and 10 µg atrazine/L for 8 weeks, no effects were reported on lymphocyte counts (Houck and Sessions 2006). In *L. pipiens* exposed to 2.1 µg atrazine/L for 21 days and then challenged with *Bd* four times over a period of 74 days, no effects were reported on biomarkers of immune function or infection and disease (Paetow et al. 2012). In a methodologically weak cosm study (SOM = 0.9) Rohr et al. (2008a) reported no effects on eosinophils in *L. palustris* exposed to 117 µg atrazine/L for 4 weeks. Exposure of *L. pipiens* to 21 µg atrazine/L for 8 days resulted in an increase in phagocytic cells (Brodkin et al. 2007). This response was judged to be beneficial (protective against infection), and a SEJ of zero was assigned.

No effects on infection or viral load were reported in a study on *A. tigrinum* challenged with *A. tigrinum* virus (ATV) following exposure to 20 and 200 µg atrazine/L for 14 days (Kerby and Storfer 2009). In the same species, the number of peripheral leukocytes following exposure to atrazine at concentrations of 1.6, 16, and 160 µg/L for 111 days with a 3-day initial exposure to ATV, was unchanged (Forson and Storfer 2006).

Other studies have reported the effects of atrazine on immune response in amphibians. In several experiments on *L. pipiens* exposed to concentrations of atrazine ranging from 0.1 to 21 µg/L, the number of white blood cells in the peritoneal cavity and phagocytic activity were reduced (Brodkin et al. 2007). However, these experiments were flawed because thioglycollate was used to elicit the cells in the peritoneal cavity and it does not recapitulate the process of an innate response mediated by any known pathogen or pathogen-associated molecular pattern. In addition, thioglycollate does not fully activate macrophages to the level at which they exhibit unambiguously increased anti-microbial activity (see SI for a more detailed discussion). There is little (if any) direct evidence that decreases in thioglycollate-elicited macrophages are associated with decreased innate immunity (i.e., decreased resistance to infection). In fact, decreased resistance to infection has been reported after administration of thioglycollate (Baker and Campbell 1980). For these reasons, the SEJ was reduced. In a methodologically weak study (SOM = 1.1) on *L. pipiens* exposed to 0.1 µg atrazine/L from 2 days post-hatch to metamorphosis, an increase in thymic plaques was reported, but significance was not tested (Hayes et al. 2006b). An increase in hemolytic plaques was reported in another weak study in *L. pipiens* (SOM = 1.4) after exposure to 1 and 10 µg atrazine/L for 4 weeks (Houck and Sessions 2006). In a study on tadpoles of *L. sylvatica* exposed to atrazine at 3 and 30 µg/L for 4 weeks and then challenged with cercariae of either *Ribeiroia sp*. or *Telorchis sp.*, the number of leukocytes in peripheral blood was reduced (Kiesecker 2002). As discussed in Solomon et al. (2008), this could have been a result of the infection, not the exposure to atrazine. For this reason, the SEJ was reduced. A series of studies on the effects of exposure of larva, metamorph, and adult *X. laevis* to atrazine at concentrations ranging from 0.1 to 10 µg/L for 7 to 30 days showed equivocal results in response to challenge with Frog Virus 3 (Sifkarovski et al. 2014). The viral copy numbers and basal expression of genes related to immune response were unaffected. Gene expression in adults was unaffected after challenge by an intraperitoneal injection (i.p.) of virus, but was increased in larvae and metamorphs by i.p and water exposures. Survival in the control and treated animals was low, and the lack of effect of atrazine on the viral copy number at all stages of development suggested that the responses may have been due to confounding stressors or poor husbandry. The score for SEJ was reduced.

In a cosm study where tadpoles were exposed to 117 µg atrazine/L (> 100 µg/L) for 4 weeks, the number of eosinophils in *L. clamitans* and the number of melanomacrophages in livers of *L. palustris* were reportedly decreased (Rohr et al. 2008a). There are always many uncontrolled variables in cosms, and this type of study is better conducted in the laboratory and this might be a response to generalized stress as suggested by Jantawongsri et al. (2013). In an ecoepidemiology study, aggregates of melanomacrophages in the livers of *L. pipiens* collected from wetlands were reported to be decreased with increased exposures to atrazine + desethylatrazine (Rohr et al. 2008a). This response was also correlated with concentration of phosphate (see discussion in section on *Indirect effects on infections of frogs…*).

A reduction in the number of peripheral leukocytes was reported in *A. tigrinum* at metamorphosis following exposure to atrazine at concentrations of 1.6, 16, and 160 µg/L for 111 days, without initial exposure to ATV (Forson and Storfer 2006). However, when challenged with virus (see above), there were no effects on leukocytes or apical endpoints. For this reason, the SEJ was reduced.

Two studies on immune function in *X. laevis* and *L. pipiens* reported a number of responses; however, these studies were based on the treatment of frogs with mixtures containing atrazine, metribuzin, aldicarb, endosulfan, lindane, and dieldrin (Christin et al. 2003). A field study in Thailand examined the incidence of melanomacrophages in the livers of *Fejervarya limnocharis* living in paddy fields (Jantawongsri et al. 2013). This study did not directly assess the effects of atrazine, as analyses for herbicides in the paddy-water were not conducted at the time the frogs were sampled. However, it was reported that atrazine, glyphosate, and paraquat were found in the tissues of frogs from the exposed site. No association was observed between residues of any of the herbicides and the incidence of melanomacrophages in frogs (Pearson's correlation, $p > 0.05$); however, melanomacrophages were more

prevalent in frogs from the exposed site in the wet season. This was not the case for the dry season. A study on a mixture of pesticides (atrazine, metolachlor, alachlor, nicosulfuron, cyfluthrin, cyhalothrin, tebupirimphos, metalaxyl and propiconazole, each at a concentration of 0.1 µg/L) on tadpoles of *X. laevis* reported damage to the thymus, and theorized that this resulted in subsequent immuno-suppression and greater incidence of infection with *Flavobacterium menigosepticum* in tadpoles and young frogs (Hayes et al. 2006b). Since atrazine was not tested alone in the above four studies, causality could not be assigned. These studies on mixtures were not scored for WoE.

Three immune-related responses to exposure to atrazine were measured in *T. s. elegans* exposed to 10 µg atrazine/L for 6 days (Polakiewicz and Goodman 2013). Pathogenic spots, swelling of the neck in turtles exposed to Ranavirus + atrazine and Ranavirus titer in turtles exposed to Ranavirus + atrazine, were unaffected.

Overall, there was little evidence of adverse effects on immune function in amphibians, fish, and reptiles exposed to realistic concentrations of atrazine (Figure 29). The mean score for strength was 1.39 ± SE 0.08, while that for relevance was 0.20 ± SE 0.06, and the null hypothesis was not falsified. Several of the experiments were poorly designed, and there is a clear need for validated experimental protocols and methods that provide clear linkages to apical endpoints before immune responses can be used in assessing risks from chemicals in the environment.

Effects of atrazine on parasites in amphibians

The studies assessed in this section are those that reported effects of atrazine on parasitism in frogs, as this might affect the apical endpoint of survival of amphibian populations. Large numbers of internal parasites might compromise growth and development. Cysts of trematode parasites can cause deformities, such as polydactyly, resulting in greater predation on and therefore reduced survival of infected frogs.

These responses were divided into those that reported direct effects on the incidence or frequency of parasitism and those that reported indirect mechanisms (i.e., ecological mechanisms) that could increase parasitism. The observation that amphibians in modified ecosystems can exhibit greater degrees of parasitism is a common, though by no means consistent, phenomenon. For example, parasitism of frogs challenged with cercariae of *Ribeiroia sp.* was reported to be greater in agricultural than non-agricultural sites (Kiesecker 2002), but the identity and concentrations of pesticides in the ponds were not reported and neither were other possible factors, such as nutrients, resulting in a reduced SEJ.

Effects of atrazine on infection of frogs by trematodes

Several studies reported no effects of atrazine on the incidence of infection of frogs by trematodes (Figure 30). The null hypothesis for this WoE assessment was that atrazine, at concentrations commonly found in the environment, does not directly alter infection of frogs by trematodes. A study of a mixture of the herbicide metolachlor and atrazine reported no effects of exposure to mixtures containing 15 and 100 µg atrazine/L with metolachlor, on rates of infectivity (Griggs

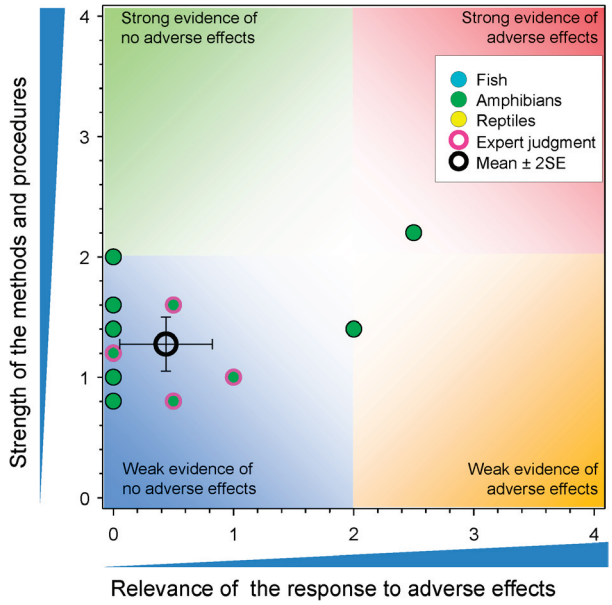

Figure 30. WoE analysis of the effects of atrazine on infection of frogs by trematodes.

and Belden 2008). This study was conducted in the laboratory and in cosms using *L. clamitans* and *L. sylvatica* tadpoles and cercariae of the trematode parasite *Echinostoma trivolvis*. Similarly, exposure of tadpoles of *L. sylvatica* to atrazine at concentrations of 3 and 30 µg/L had no effect on parasitism by cercariae of *E. trivolvis*, when both were exposed to atrazine (see below (Koprivnikar et al. 2007)). In addition, laboratory exposures of *L. clamitans* to atrazine alone, at 201 µg/L, had no statistically significant effect on infections by cercariae of *E. trivolvis* (Rohr et al. 2008b). Reductions (a beneficial effect) in infection by Bd were reported in tadpoles of *O. septentrionalis* exposed to 1.06, 10.6, 58, and 106 µg atrazine/L for 28 days in the laboratory (McMahon et al. 2013). However, in the same species exposed to 66 µg/L in cosms, resistance to challenge by Bd was unaffected while tolerance decreased. The SEJ for this latter response was decreased because of high mortality in the control and treated animals.

Other experiments have produced different results. A cosm study with tadpoles exposed to 117 µg atrazine/L (> 100 µg/L) and *Plagiorchid* cercariae, reported increased infections and significant changes in markers of immune responses (e.g., melanomacrophages) in *L. clamitans* and *L. palustris,* compared to the controls (Rohr et al. 2008a); however, the numbers of cercariae in control and treated cosms were not enumerated and could have been a confounding variable. Moreover, the response of other amphibians introduced (5 individual *A. maculatum* and 20 individual *H. versicolor*) to atrazine was not reported, which can be inferred to mean either a result of no effects on parasitism, or a significant loss of individuals due to poor husbandry in the cosms. In another study, infectivity of *E. trivolvis* in tadpoles of *L. sylvatica* was increased (more cysts were observed per individual, but there was no increase in the number of tadpoles infected) after exposure to 30 (but not 3) µg atrazine/L, if the tadpoles, but not the cercariae, were exposed (Koprivnikar et al. 2007). Since host and parasite would both be exposed simultaneously in the environment, this is an unlikely scenario, resulting in a

reduced SEJ being assigned. Conversely, infectivity of cercariae of *E. trivolvis* exposed to 20 and 200 µg atrazine/L in the presence of unexposed tadpoles of *L. pipiens* was significantly decreased relative to controls (Koprivnikar et al. 2006). Because this response was not adverse (but still significant), a SEJ of zero was assigned. Laboratory-exposures of tadpoles of *L. sylvatica* to atrazine at concentrations of 3 and 30 µg/L for 28 days, followed by exposure to *Telorchis* and *Ribeiroia* spp., resulted in increased rates of infection compared to controls with no exposure to atrazine (Kiesecker 2002).

An ecoepidemiology study of trematode infections in frogs exposed to concentrations of atrazine ranging from 0.075 to 0.6 µg/L reported an association between the rates of infection and concentration of atrazine and its metabolite, DEA (Rohr et al. 2008a). The ecoepidemiology component of this paper was based on data from an earlier study in 1999–2001[1]. In this three-year study, about 72 sites in IL, WI, and MN were investigated and characterized. Specific to the trematode parasites, a PhD thesis based on the work (Schotthoefer 2003) concluded that "The distributions and abundances of parasites across populations were related to the availability of forest at distances within 2 km and up to 10 km distances from wetland perimeters. As more forest was available, mean parasite abundances and richness of parasite communities increased. In contrast, frogs that emerged from wetlands within landscapes dominated by agriculture, where forested habitats were much more limited, tended to be infected with fewer parasite species, at lower abundances". This is contradictory to the conclusions of the ecoepidemiology study by Rohr et al. (2008a) that was based on a subset of the same data. To explain the role of atrazine in the stimulation of parasitism, Rohr et al. (2008a) proposed that the increased rates of infection resulted from the stimulation of periphyton and the repression of phytoplankton, which caused the snails, hosts of the trematode parasites, to increase in number, resulting in greater loads of parasites. This is an indirect mechanism and is discussed in more detail in the next section on *Indirect effects of atrazine on parasitism of amphibians*.

The mean score for strength was 1.34 ± SE 0.12, while that for relevance was 0.44 ± SE 0.19, and the null hypothesis was not falsified. Few studies were considered very strong, and this is partly due to the challenges of investigating the role of a contaminant in modifying a parasite-host relationship, especially under laboratory conditions (Marcogliese and Pietrock 2010). Moreover, the ecological implications of subtle changes in background parasitism rates for individuals are not well understood.

Indirect effects of atrazine on parasitism of amphibians

The null hypothesis for this WoE assessment was that atrazine, at concentrations commonly found in the environment, does not indirectly affect parasitism of amphibians. A number of studies (42 responses) have been conducted to characterize effects on parasitism indirectly caused by atrazine. The first group of these studies is based on the effects on algae in surface waters. This theory postulates that atrazine is more toxic to phytoplankton than to periphyton, and that the latter will increase in biomass when atrazine is present in the system (Figure 31). The increase in biomass of periphyton favors the snails (intermediate hosts for trematodes) that graze on this resource, and this ultimately results in increased parasitism in frogs due to a larger intermediate host pool. This simple model has been demonstrated for nutrient inputs (Johnson et al. 2007), and several studies have investigated the potential role that atrazine may play in favoring the growth of snails via this mechanism. However, studies to date have provided no strong evidence in support of these predicted mechanisms, and most evidence indicates that the proposed cascade is unlikely for atrazine.

In methodologically robust cosm studies (SOM = 3) treated with atrazine at concentrations ranging from 1 to100 µg/L, no effects were reported on biomass (ash-free dry wt.) of periphyton and phytoplankton over 70 days (Baxter et al. 2011). Decreases in biomass of macrophytes were observed in cosms treated with 100 µg atrazine/L, but there were no effects on other endpoints (see above and following) (Baxter et al. 2011). Moreover, the loss of macrophytes would likely result in a decrease in the abundance of snails due to their utilization of macrophytes as a food source, along with periphyton.

In a weak study (SOM = 0.9) in cosms treated with 50 µg atrazine/L, the biomass of periphyton, measured as chlorophyll-a (Chl-a), was reported to decrease significantly (Rohr and Crumrine 2005). Although significant, the SEJ was reduced, as the decrease in periphyton would not trigger the postulated cascade leading to increased numbers of snails. In another study (SOM = 0.9), it was reported that in cosms treated with 117 µg atrazine/L (> 100 µg/L), periphyton increased in biomass as phytoplankton decreased (both measured as Chl-a) and clarity of water increased (Rohr et al. 2008a). In another cosm study (SOM = 1), Chl-a in phytoplankton decreased

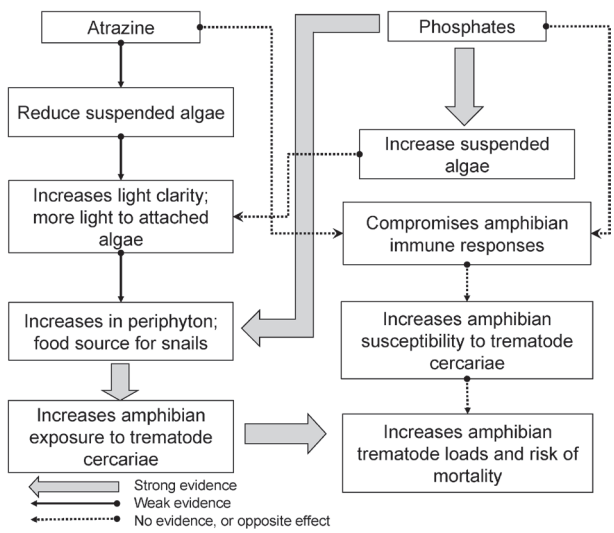

Figure 31. Conceptual model of the proposed steps by which atrazine and phosphorous can increase trematode infections in amphibians in small lentic systems. The type of the arrow indicates the strength of evidence for a causal link between each step related to atrazine or phosphorous at environmentally relevant concentrations. Modified from Figure S4 in (Rohr et al. 2008a).

[1]See three interim and final reports on the project of Beasley et al. "Environmental Factors That Influence Amphibian Community Structure and Health as Indicators of Ecosystems" at http://cfpub.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/274/report/F

after exposure to atrazine at a concentration of 200 µg/L for 57 days (Boone and James 2003).

Snails are an important component of the postulated cascade of effects, whereby atrazine could increase parasitism in frogs. No effects were observed in uninfected snails (*Stagnicola elodes*) exposed to de-ethyl atrazine (DEA), a degradate of atrazine, at a concentration of 0.33 µg/L (Koprivnikar and Walker 2011); however, mortality increased significantly in exposed snails infected with trematodes. A SEJ of zero was assigned for the decrease in snails because the effect would not be detrimental to frogs. In cosms treated with 201 µg atrazine/L (Rohr et al. 2008b) and 50 µg atrazine/L (Rohr and Crumrine 2005), the survival of snails (*Planorbella trivolvis*) was unaffected. The mass of snails and number of egg-masses in cosms treated at 50 µg atrazine/L were reduced (Rohr and Crumrine 2005), which is contrary to what was seen in the cosm study at 117 µg atrazine/L (Rohr et al. 2008a). The SEJs for these responses were reduced because responses were inconsistent with the postulated cascade, leading to increased parasitism. The number of egg masses of snails were unchanged by exposures to atrazine at 201 µg/L (Rohr et al. 2008b). The number of snails (*Physella* spp and *S. elodes*), mass of snails, number of egg-masses, and number of eggs per egg-mass were all unaffected in cosms treated with atrazine at concentrations of 1, 10, 30, and 100 µg/L (Baxter et al. 2011). In another cosm study, treatment with 117 µg atrazine/L resulted in increases in the number of snails hatching from eggs and the number of snail eggs per cosm (Rohr et al. 2008a).

Several studies have reported that atrazine adversely affected the survival of cercariae, which is again the opposite of the postulated cascade to increased parasitism. No increase was seen in the release of cercariae of *Echinoparyphium* sp. from *S. elodes* treated with 0.33 µg DEA/L (Koprivnikar and Walker 2011). Three other studies demonstrated significantly reduced survival of cercariae of trematodes exposed to concentrations of atrazine ranging from 2 to 2,000 µg/L (Griggs and Belden 2008, Koprivnikar et al. 2006, Rohr et al. 2008b). SEJs of zero were assigned to all of these studies because the response was inconsistent with the postulated cascade, leading to increased parasitism. In laboratory studies on Bd, McMahon et al. (2013) reported the lack of growth (beneficial effect) at exposures to atrazine at concentrations ranging from 0.011 to 216 µg/L.

The mean score for strength was 1.75 ± SE 0.14, while that for relevance was 0.02 ± SE 0.01, and the null hypothesis was not falsified. The distributions of the individual scores (Figure 32) do not support the three lines of evidence (namely, shifts in production from phytoplankton to periphyton, increase in productivity in snails, and greater numbers/activity of cercariae) that are needed to corroborate the postulated cascade of effects leading to increased parasitism. When coupled with the weak association between atrazine and direct effects on parasitism (described above), this further weakens support for the postulate of the trophic cascade. This is also consistent with the conclusions of others who have noted that, while periphyton are somewhat less sensitive than phytoplankton to atrazine and other photosynthesis-inhibiting herbicides (Brock et al. 2000), this has only been observed at large concentrations (≥ 100 µg atrazine/L) (Herman et al. 1986). In flowing wetland mesocosms, atrazine, at concentrations ranging from 15 to 75 µg/L, had various effects on periphyton (Detenbeck et al. 1996); gross productivity of periphyton was reduced at 15 µg/L, but Chl-a and ash-free dry weight were unaffected at the greatest concentration tested (75 µg/L). In a study of stream mesocosm, exposure to atrazine at 25 µg/L for 10 months resulted in no changes in the biomass of periphyton (Lynch et al. 1985).

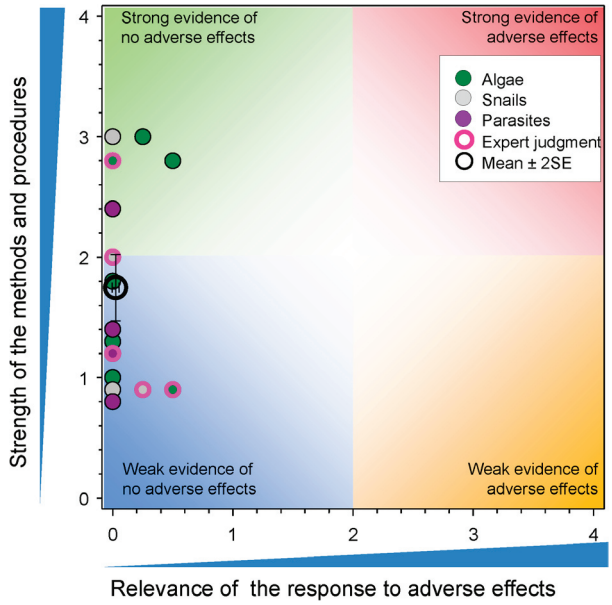

Figure 32. WoE analysis of the indirect effects of atrazine on infection of frogs by trematodes.

Nutrients have been shown to be more important drivers of growth in periphyton and phytoplankton, than atrazine (Baxter et al. 2013). As nutrients, especially phosphorous, are known to be drivers of the trophic cascade leading to increased parasitism (Johnson et al. 2007), this provides a more plausible hypothesis that nutrients were the drivers of effects observed in the field (Rohr et al. 2008a). As has been pointed out (Rohr et al. 2012), the small concentrations of atrazine (0.075 to 0.6 µg/L) measured in wetlands (Rohr et al. 2008a) at the time of sampling may merely have been markers of earlier inputs of nutrients via run-off that had already been assimilated and stimulated the trophic cascade, leading to the observed increase in parasitism in this subset of wetlands. The co-occurrence of nutrients and pesticides, including atrazine, in field run-off, has been observed in a number of agricultural settings (Andrus et al. 2013, Dalton et al. 2013). As noted in the original work that postulated the link, phosphorous was also strongly associated with the observed increase in parasitism (Rohr et al. 2008a), but as noted by Baxter et al. (in response to a letter from Rohr et al. 2012), sampling at inappropriate times might have under-estimated the correlation of this nutrient with observed levels of parasitism. Agricultural activity itself has been linked to increased parasitism of amphibians by some trematodes (Koprivnikar and Redfern 2012), most likely because of greater inputs of nutrients, and not pesticides in general (Skelly et al. 2010).

Direct effects of atrazine on behavior

Although behavior of organisms is important for individual survival, the linkage between changes in behavior, espe-

cially short-term modifications, resulting from exposures to chemicals in the environment and apical endpoints such as survival, have not been clearly identified. The null hypothesis for this WoE assessment was that atrazine, at concentrations commonly found in the environment, alters the behavior of fish, amphibians and reptiles. Studies with several species of fish (31 responses), amphibians (27 responses), and reptiles (5 responses), have been conducted specifically to examine the potential effects of atrazine on behaviors such as avoidance, mating, or response to predatory cues. These behaviors are associated with potential water loss, mating, migration, or social interactions important to maintaining populations. Behavioral responses, such as the ability to maintain equilibrium, have also been reported in other studies as indicators of sublethal toxicity. Studies vary widely in behavioral endpoints, exposure concentrations, and duration of exposure, the latter ranging from a few minutes to several weeks. The results of the WoE assessment are shown in Figure 33.

Fish

In behavioral trials with *O. mykiss*, no avoidance or preference to the solvent control (acetone) or atrazine (10 and 100 µg/L) was observed after a 10-minute exposure (Tierney et al. 2007). In a second trial, after exposure to atrazine at concentrations of 1, 10, and 100 µg/L for 20 minutes, fish exhibited a significant increase in locomotion at atrazine levels of 1 and 10 µg/L only, and in contrast, avoidance was only significantly different at 10 µg/L. The SEJ for the latter response was reduced due to lack of consistent concentration-response and the transitory nature of the response. Saglio et al. (1998) exposed juvenile *C. auratus* for 24 hours to atrazine at concentrations of 0.5, 5, and 50 µg/L, and monitored behaviors such as sheltering, grouping, surfacing, and burst-swimming. As the behavioral responses were generally not significant, or were inconsistent between very high and low concentrations, the SEJ was reduced. This trial was repeated, to include observations after 24 hours in clean water, and no significant effects were observed following exposure to atrazine, indicating that any observed effects would be transitory. A third trial included exposure to skin extract of *C. auratus* (alarm cue) post-atrazine exposure, and these fish exhibited a significant difference for sheltering and grouping, but only at the middle concentration. Again, due to inconsistencies between high and low concentration-responses and also the large differences between control responses, the SEJ was reduced.

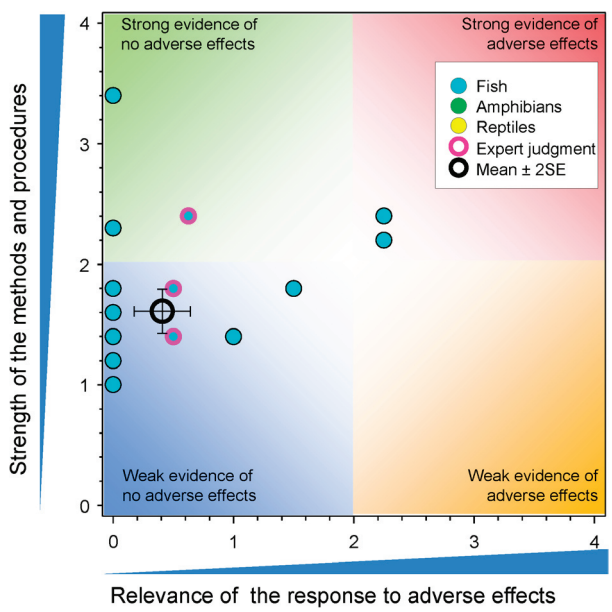

Figure 33. WoE analysis of the effects of atrazine on behavior in fish.

Behavioral observations of *L. macrochirus* and *P. promelas* (disequilibrium) at atrazine concentrations of 1,000 µg/L (Mehler et al. 2008), *S. salar* (eating, swimming, schooling, and stress) at 1 to 100 µg atrazine/L followed by a SW challenge (Matsumoto et al. 2010), and *S. ocellatus* at 40 and 80 µg atrazine/L (startle response, predator response) (del Carmen Alvarez and Fuiman 2005), were not significantly different from those of the controls. The latter study, however, reported increased activity related to swimming, after 4 days at both exposure concentrations. The SEJ was reduced because there were no effects on the apical endpoint of survival and the reduction in growth rate after 9 days of exposure was slight (~9%). A loss of balance was reported in an acute study with *M. fluviatilis,* but this occurred at large concentrations (EC50 values $>$ 8,000 µg atrazine/L). In a chronic study (28 days duration), the feeding behavior of *D. rerio* at 90 µg atrazine/L corresponded to a significant reduction in growth rate (Plhalova et al. 2012). Although the response was not quantified and no statistical tests were performed, it was assumed to be significant, at 90 µg atrazine/L.

The effects of atrazine (1 or 15 µg atrazine/L for 16 weeks) on the reproductive behaviors of *P. reticulata* were evaluated (Shenoy 2012). This author reported a significant reduction in male mating displays for the combined atrazine treatments; however, no effect on mating attempts was observed, and therefore, there appeared to be no relevance. When solvent control males were paired with atrazine-treated males, no statistically significant differences were observed for proximity to female, number of mating attempts, and interaction with opponent`s behavior. Exposure to atrazine at 15 µg/L, but not at 1 µg/L, resulted in significantly fewer rival attacks and aggressive displays, in comparison to solvent control. The relevance of reduced aggression, as related to measures of reproductive success or egg production, was not characterized in the study.

Behavioral studies in *D. rerio* exposed to atrazine at concentrations of 10 and 1,000 µg/L for 14 days showed a number of changes in responses such as size of shoal, inter-fish distances, social interactions, and depth preference (Schmidel et al. 2014). However, these response were only observed at the large concentration of 1,000 µg atrazine/L.

Moore and colleagues have published a number of studies on the potential effects of atrazine on *S. salar*, which have included behavioral observations. Moore et al. (1998) reported a significant reduction in the detection of the female pheromone, PGF2α, by the olfactory epithelium of adult male salmon after a 30-minute exposure to atrazine at concentrations of 2, 5, 10, or 20 µg/L. It is not clear if habituation occurred or if this would actually affect mating. In a later study (Moore and Lower 2001), they reported a reduction in the male olfactory response to PGF2α after a 5-day exposure to 1 µg atrazine/L. The authors state "Atrazine is a known inhibitor of acetylcholinesterase (AChE) activity"

and that this is a possible mechanism of action; however, this mode of action is incorrect and is not supported by available studies. In another study, Moore et al. (2007) reported significant reduction in the olfactory response to L-serine and smolt urine after *S. salar* smolts were exposed for 5 days to 0.5, 1.0, 2.0, and 5.0 µg atrazine/L. Atrazine alone did not elicit an electro-olfactogram (EOG) response. The relationship between altered olfactory responses and apical responses, such as survival or reproduction, is not known. These authors also tested the nocturnal migratory activity of the salmon smolts following an 81-day exposure to 0.5 and 5.0 µg atrazine/L in FW and subsequent transfer to SW. There was a significant decrease in the migratory activity within the 4,000-L tank over the 28-day study period, at 5.0 µg atrazine/L, compared to controls. There was no significant difference between the groups in the periodicity of the migratory activity, i.e., the mean times for the initiation of migration at night, or times the fish were last recorded migrating each night. The relevance of the reduction in only one of four migratory behaviors is not known. Moore et al. (2008) also monitored the movement of salmon smolts from FW into the marine environment following a 72-hour exposure to 0.1 µg atrazine/L. Smolts from the control and atrazine-exposed groups successfully migrated across the FW section of the study site, and there was no significant difference in the mean time taken to migrate across the bay.

The functionality of olfactory sensory neurons, as measured by EOG responses, and the activity of the detoxification enzyme GST, were measured in *O. mykiss* after exposure to a mixture of pesticides including atrazine (Tierney et al. 2008). Atrazine was not tested alone, so there was no indication of whether atrazine was responsible for any of the changes measured.

The mean overall score (Figure 33) for strength of the studies of behavioral effects of atrazine in fish was 1.61 ± SE 0.09, while that for relevance was 0.41 ± SE 0.12. The null hypothesis was not falsified.

Amphibians

The results of the WoE analysis of behavioral responses to atrazine in amphibians are summarized in Figure 34. Larval *L. pipiens, L. sylvatica,* and *B. americanus* were exposed to atrazine at concentrations ranging from 2,590 to 20,000 µg/L, at 96 hours post-hatch, and no effects on swimming speed were observed (Allran and Karasov 2001). A behavioral study was conducted, with *B. americanus* tadpoles that were exposed to 201 µg atrazine/L for 4 days and observed for response (location and activity) to various cues (Rohr et al. 2009). There were no significant differences between responses of atrazine-treated tadpoles and control tadpoles to the presence of food, a predation cue, the parasitic cercariae of *E. trivolvis*, and a snail releasing cercariae of *E. trivolvis*, or an uninfected snail. The number of tadpoles of *L. sylvatica* hiding or avoiding a caged predator during 4 weeks of exposure to a maximum of 50 µg atrazine/L in small outdoor cosms was reported in the study (Rohr et al. 2004). There was a small (~15%) reduction in hiding behavior and no effect on predator avoidance. The SEJ was reduced as the relevance of the adverse effect in frogs was not characterized.

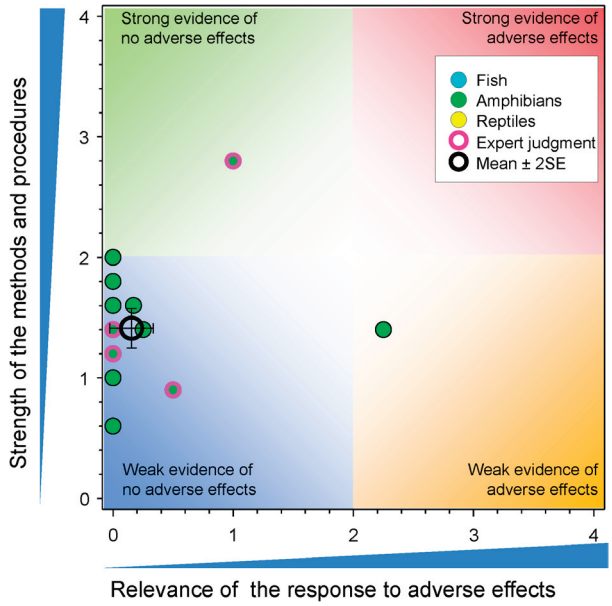

Figure 34. WoE analysis of the effects of atrazine on behavior in amphibians.

Metamorphs of *B. americanus* were assessed for 2.5 days in the laboratory, for the avoidance response to atrazine-spiked soils (80 or 1,430 µg/kg). There were no statistically significant choice preferences for spiked soils (Storrs Mendez et al. 2009). However, for one or two of the six timeframes assessed, there were significantly more frogs on atrazine-treated soil than on untreated control soil. The SEJ was negated because the lack of avoidance is not adverse for survival, especially in light of any evidence of acute toxicity at these concentrations. There was no effect on the feeding rate of *X. laevis* tadpoles exposed to 25 and 200 µg atrazine/L from NF stages 47 to 63, and a significant increase was reported for a weekly observation only at an atrazine concentration of 400 µg/L (> 100 µg/L) (Zaya et al. 2011b). In a sexual development study, abnormal swimming in developing tadpoles of *X. laevis* exposed to atrazine at concentrations of 1, 10, and 25 µg/L, was reported at the highest concentration only (Carr et al. 2003). The increased frequency above controls was only 2.4% at 25 µg/L, and it had no effect on survival or size at metamorphosis, so a reduced SEJ was assigned.

A series of related studies (Rohr et al. 2003, Rohr et al. 2004, Rohr and Palmer 2005, Rohr et al. 2006, Rohr et al. 2013) have reported on the behavioral responses of the salamander *A. barbouri* to atrazine exposures (see SI for study sequence). Rohr et al. (2003) reported no significant difference in the percentage of larvae in refuge or moving in a 30-second period, in the presence or absence of food, after exposure to atrazine at concentrations of 4 and 40 µg/L, from 2 to 39 days post-fertilization. However, there appeared to be a monotonic decrease in refuge use in animals exposed to 400 µg atrazine/L. This study also reported a significant increase in movement (only in the presence of food) at the large exposure concentration of 400 µg/L atrazine only (> 100 µg/L). The authors suggested that atrazine caused, "underlying nervous system malfunction," but the mode of action was not investigated in specific experiments, and there were no observations from other studies to suggest

that atrazine acts on the nervous system at these exposure concentrations.

In the second study of the series, Rohr et al. (2004) reported significantly fewer salamanders in refuge after exposure to 400 μg/L atrazine from Harrison-stages 18–28, to metamorphosis. The absolute differences from controls were slight (5.5%–5.6%), of questionable biological significance, and occurred at a concentration greater than 100 μg/L. No differences were observed at 4 or 40 μg/L.

In the third paper of the series, salamanders exposed in the 2004 study were reared to 137–139 days (early ontogeny - EO) or 237–239 days (late ontogeny - LO) after metamorphosis and their last atrazine exposure, and behavioral observations related to potential water loss were reported (Rohr and Palmer 2005). The animals were tested individually (singlets) or in groups of three (triplets), and there was a significant decrease in the inactivity in singlets (EO) exposed to atrazine at 400 μg/L and (LO) at 40 and 400 μg/L. No similar change in the degree of inactivity was observed when the animals were tested as triplets. A measure that is likely the inverse of activity, was use of the side of the Petri dish. A significant decrease was observed for singlets (EO), at 400 μg/L only, and no effects at any concentration for LO animals. As with the measure for inactivity, no effects were observed for use of the side of the Petri dish when the animals were tested as triplets. Testing single individuals, rather than groups, clearly influences the response, and the significance of this, as it relates to risk in the environment, was not addressed by the authors. Also, it should be noted that no mortality occurred in these animals for up to 239 days after atrazine exposure, which questions the relevance of the differences reported in behavioral observations with respect to apical endpoints. The fourth publication in the series utilized additional animals exposed, as in Rohr et al. (2004) (4, 40, or 400 μg/L atrazine from Harrison-stage 28 to metamorphosis to 122 days), and then observed for 77 days under different moisture and temperature combinations (Rohr and Palmer 2013). A number of behaviors such as huddling, inactivity, refuge use, burying during foraging, and foraging rate were reported to be affected by prior atrazine exposure. However, the study apparently suffered from very serious husbandry issues, as demonstrated by the fact that even control animals lost weight during the 77-day study, and mortality across treatments appears to have been 60% – 80%. This mortality would have resulted in reduced and unequal treatment observations, and assessing normal behaviors in weak and dying animals is inappropriate. The SEJs for these responses were assigned a value of zero. In a study on locomotory performance of *A. maculatum* after a dermal exposure to atrazine at concentrations of 27.1 and 271 μg/L for 24 hours, no effects were reported (Mitchkash et al. 2014). The mean score for strength of the studies in amphibians was $1.41 \pm$ SE 0.08, while that for relevance was $0.15 \pm$ SE 0.09, and the null hypothesis was not falsified.

Reptiles

One study has included behavioral observations of reptiles (WoE scores not graphed). Neuman-Lee et al. (2011) exposed the eggs of *G. ouachitensis* and *G. pseudogeographica*, via sand drench, to atrazine concentrations of 0.1, 1, and 100 μg/L. In the first week after hatching, the turtles were tested for righting time and swimming. The turtles were reared for a further 11 months and tested for time to first feed, escape behavior, and forage time. No significant differences were observed between any control and atrazine-treated turtle behaviors, and a WoE diagram was not constructed. The mean score for relevance was $0 \pm$ SE 0, the mean score for strength was $1.6 \pm$ SE 0, and on the basis of the single study, the null hypothesis was not falsified.

In general, the SOM for these behavioral studies with fish, amphibians, and reptiles were low (SOM < 2, Figure 33 and Figure 34), especially for studies specifically designed for assessment of behavioral endpoints. Studies often suffered from 1) lack of adequate replication, 2) pseudo-replication, 3) small sample sizes, 4) unblinded observations, and/or 5) monitoring behavior in weakened animals. The relevance of monitored behaviors relative to apical endpoints was not always clear. Behavioral responses were often observed at concentrations greater than 100 μg/L. Overall, these studies provide weak to moderate evidence of no adverse effects on behavior by atrazine.

Effects of atrazine at the level of the population

Since the last review (Solomon et al. 2008), there are still only limited data on the potential relationship between atrazine and changes at the population-level for amphibians, fish, and reptiles, upon which to conduct an evaluation. Studies on populations are carried out in the field, and as in human epidemiology, it is difficult to account for confounding stressors and exposures to multiple compounds. This makes it difficult to assign causality, except to infer from the lack of response that atrazine, along with other stressors that co-occur, is not the cause of noticeable declines in populations. The specific effects of interest include changes in the abundance of species, richness and evenness, reproductive fitness, and other metrics directly related to the status of the population (or community). By definition, these metrics can only be effectively measured at the field-level (or long-term cosms studies), which is difficult due to logistical restraints and confounders, such as the presence of pathogens or other contaminants (Beebee and Griffiths 2005). No adverse effects on amphibians and fish have been observed in a number of mesocosm and microcosm studies, except when treated at high concentrations, which caused indirect effects via an alteration of aquatic macrophytes and habitat, or a reduction in food supply for grazing fish (Solomon et al. 2008).

Although no studies that specifically tested the effects of atrazine on fish at the population level were found in the literature, a few field studies have assessed responses of fish to atrazine (and other stressors), and some inferences can be made. The studies on *M. dolomieu* and *M. salmoides* in the Potomac River basin used fish collected in several locations with different inputs of chemicals into the water (Blazer et al. 2012, Iwanowicz et al. 2009, Kolpin et al. 2012). Although not stated in these papers, it is inferred that sufficient fish were present to sample and that populations had not been decimated by atrazine or the other stressors, either individually or in combination.

Most studies examined potential linkages between amphibian population or community status and the influence of agriculture in general. For example, historical pesticide use in California, specifically OP insecticides (atrazine did not appear to be part of the assessment), have been correlated with population declines of ranid, but not non-ranid frogs (Davidson 2004). However, it is unclear from the study how the influence of the changing and expanding agricultural landscape, which would be correlated with expanded pesticide use in the region, was accounted for.

Some studies have reported on the relationship between agricultural activity and populations of amphibians. Knutson et al. (2004) found no differences in the richness of species or the reproductive success of amphibians in natural wetlands versus those associated with row-crop agriculture (corn or soybeans) in Minnesota, USA where atrazine (<0.1– 0.5 μg/L) and di-ethyl atrazine (<0.1– 0.3 μg/L) were detected. They did observe significant negative associations between nutrients and reduced reproductive success (phosphorous) and species richness (nitrogen), typically as a result of livestock activity, providing evidence for the role of nutrients as the primary driver of observed impacts (see section on *Effects of atrazine on parasites*). Earlier work by the same group (Knutson et al. 1999) found a positive association with agricultural cover and the abundance of amphibians in Wisconsin, but no association in Iowa, though crop cover and water quality parameters were not measured. Similar findings were found in Ontario, Canada where nutrients (phosphorous, ammonia, and TKN) were negatively correlated with anuran diversity and density in an intensive agricultural setting (Bishop et al. 1999). Atrazine was measured as part of the study and found in the surface water (0.06 – 6.47 μg/L), but the study did not report at what sites (upstream versus downstream), nor if there was any association to responses in the amphibians, so it was not possible to score for WoE. Du Preez et al. (2005b) sampled natural populations of *X. laevis* from corn-growing areas where atrazine was used historically and at the time of sampling, and from non-corn-growing areas in South Africa, and found no differences in sex ratios, abundances, or age-structure of collected animals. Hayes et al. (2003) examined the relationship between exposure to atrazine in the field (corn- versus non-corn-growing regions) and presence of gonadal dysgenesis and TOFs in 100 *L. pipiens* collected across each of seven sites. In this study, no mention was made of any problems in finding and capturing this number of individuals in the different locations, implying that the populations were sufficiently healthy to allow this level of capture. Similarly, Christin et al. (2013) collected young-of-the-year *L. pipiens* from reference and agriculturally-impacted sites in Quebec, Canada, and reported no difficulties in obtaining animals, with atrazine detected at all sites (concentrations not reported, so causality could not be assessed).

Although the data are still limited, the published literature does not make a compelling case for population- or community-level effects related to atrazine exposure in amphibians. While further studies might be needed before any final conclusions about population- or community-level effects of atrazine on amphibians can be reached, it is simply more likely that the observed local and global declines in amphibians are not related to atrazine, but the combined pressures of habitat loss, invasive species, pathogens (fungal and viral), and climate change, amongst a myriad of ongoing stressors (discussed below).

If atrazine were a significant contributor to amphibian declines, we would predict that, in regions where declines have occurred, and atrazine is no longer used, there should be a cessation or a reversal in the fate of affected organisms. This is not the case, as illustrated by the continuing plight of amphibians in the European Union, where atrazine has not been registered for use since 1991 in Italy and Germany, and since 2004 in the entire European Union. As of 2009, the International Union for the Conservation of Nature reported that 25% of European amphibians are threatened, with 17% near threatened, and habitat loss as the major driver (Temple and Cox 2009a). The general population trends for amphibians in Europe are that 59% of species are continuing to decrease, with 2.4% increasing, and the rest unknown (2.4%) or stable (36.1%). The cessation of atrazine use in much of this region does not appear to have had any impact on the status of these vulnerable species. Similar trends hold for reptiles in the same regions, with 19.4% threatened, and 12.9% near threatened, and habitat loss, fragmentation, and degradation identified as the major drivers (Temple and Cox 2009b). Overall, 41.7% of reptile species in Europe are continuing to decline, 41.7% are considered stable, and the rest are unknown (13.7%) or increasing (2.9%).

The continuing and expanding declines in amphibians globally, regardless of atrazine use in the areas of observation, have been an ongoing concern for herpetologists and conservation biologists for several decades now. The general consensus in the conservation community is that habitat loss, disease, invasive species, and climate change, or interactions amongst these stressors, are the driving forces behind the phenomena. Of these, the most insidious is the fungal pathogen *Bd*, which causes chytridomycosis and is spreading rapidly around the world and is directly linked to severe declines or complete loss of rare and endemic amphibians. It has been found in 52 of 82 countries where samples have been analyzed, and in 516 of 1240 species where testing has occurred (Olson et al. 2013). It continues to be identified as a causal factor in losses of amphibians from habitats, both pristine and modified, from around the world. In addition, it has been suggested that more diverse and more complex amphibian communities appear to be at greater risk of losses due to *Bd* infections (Olson et al. 2013).

Habitat loss is also a key driver of amphibian declines globally (Beebee and Griffiths 2005), especially wetland loss, as noted by the IUCN in Europe (Temple and Cox 2009a). For example, reductions in populations of *R. temporaria* in Ireland are mainly related to the availability of habitat and the ongoing draining of agricultural ponds, not contaminants (Reid et al. 2013). Habitat loss appears to be the key driver in Africa, where *Bd* is likely endemic due to its association with *X. laevis*, and habitat loss has been linked to the decline in frog species in Ethiopia (Gower et al. 2013).

Invasive species are also directly linked to amphibian declines. The introduction of bullfrogs has been linked empirically to adverse effects on survival of the California red-legged frog (*Rana aurora draytonii*) (Lawler et al. 1999). In a whole-lake field study, Vredenburg et al. (2004) demonstrated that the introduction of rainbow and brook trout

were responsible for declines in mountain yellow-legged frog (*Rana muscosa*) in lakes of Sierra Nevada, and that, upon their removal, the frog populations recovered rapidly (Knapp et al. 2007).

Increasingly, climate change has been implicated in amphibian declines, primarily as it relates to changes in breeding patterns (Beebee and Griffiths 2005). Still, in many cases, it might be a combination of one or more of these factors (disease, habitat loss, climate change, etc.) that maybe pushing species into decline, as has been postulated for woodland salamanders in Eastern North America [disease and climate change] (Caruso and Lips 2013), or *Bombina pachypus* in Italy [disease and habitat loss] (Canestrelli et al. 2013), or the Genus *Atelopus* in Central and South America [disease and climate change] (Rohr and Raffel 2010). An interesting example is in the Sierra Nevada mountains, where introduced species threaten the sustainability of the frog population (mentioned above), but where *Bd* is also implicated in amphibian declines. In these lakes, *Pseudacris regilla* appears to be resistant to *Bd*, and acts as a reservoir to infect vulnerable species (Reeder et al. 2012), illustrating the complex nature of the problem when trying to attribute causation, or initiate conservation efforts.

Discussion and conclusions

The overall summary of the WoE analyses for all the responses that were graphed in the above sections is shown as the mean and 2 × SE for relevance and strength of each response (Figure 35). The important point illustrated in this complex graphic is that all the means cluster on the left side of the graphic, indicating weak to no relevance of the responses to adverse effects. They also cluster in the center of the Y-axis, indicating moderate strength of the studies on average. The SE bars indicate that there is greater uncertainty for some responses than others, this largely because of lack of number of observations, but occasionally because of bimodal scores for relevance where different responses to atrazine were observed in Atlantic salmon from the western and eastern shores of the Atlantic ocean (see section on *Smoltification of fish*…). This might be related to the underlying experimental design, the strain of salmon, the age of the smolts, or the type of SW challenge (e.g., direct transfer to full SW within a short period or via a series of graded concentrations).

Each of these mean values represents one response, and is discussed in more detail in the preceding text. However, these responses can be linked in lines of evidence. As illustrated in Figure 33, lines of evidence can be assessed in two ways. In some cases, the responses are the actual apical endpoints that are the protection goals, i.e., successful development or reproduction. These are independent and the sum of their strengths and relevance provides the total weight of evidence. Other endpoints can be concatenated into a single line of evidence where the responses are dependent. Concatenation of responses in this way is referred to as an Adverse Outcome Pathway (AOP, Figure 36), such as is described in Ankley et al. (2010). In this case, there is an initiator response, usually at the level of the receptor. As a result of this response, the effect can propagate to the next level of organization and cause a response in one or more organs. This then propagates to organisms and/or the population where the apical endpoints (survival, development, reproduction, and growth) may be affected. Because these lines of evidence are dependent, the total weight of the evidence is the product of the individual responses. If these are expressed in terms of probabilities, the total weight is the likelihood that there is a causal link between the stressor and one or more apical endpoints.

If a relevant effect(s) on an apical endpoint is observed and appropriate studies are available, it is possible to develop an understanding or explanation of the mechanisms at lower levels of organization. This understanding might facilitate extrapolation to other organisms/taxa or identify reliable and robust biomarkers that can be used in place of the apical endpoint. If the only studies available are at lower levels of organization, the AOP can be used to design hypothesis-testing experiments

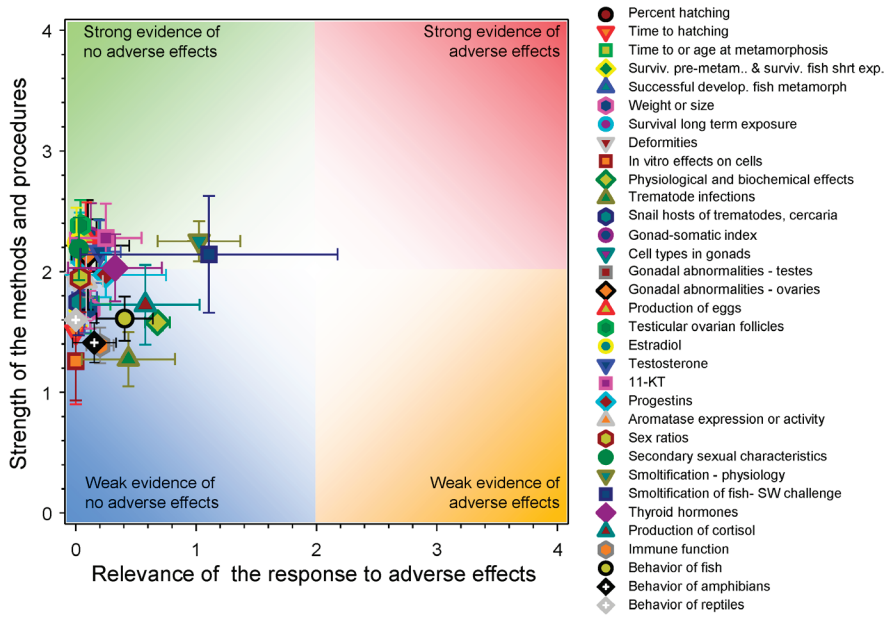

Figure 35. Overall summary of all WoEs. Symbols are means and horizontal and vertical bars indicate 2 × SE.

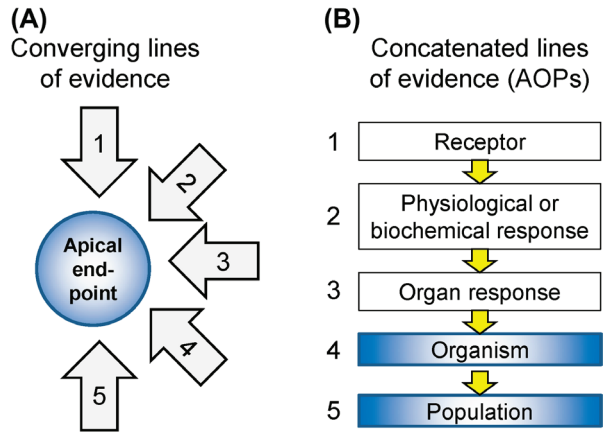

Figure 36. Illustration of converging lines of evidence (A) and concatenated lines of evidence on an adverse outcome pathway (B).

at higher levels of organization. If one or more apical endpoints have been assessed under WoE, and the combination of these indicates none or *de minimis* effects, an analysis of AOPs is not needed. However, if one or more of the apical endpoints indicates relevant effects, then a characterization of AOP might be useful to better understand the response.

Organisms respond to a stressor by adaptation. Provided that the energy and other costs of adaptation to the stressor do not compromise the apical endpoints in an organism, these adaptations are not adverse; they are merely expression of resiliency. Initially, adaptation to stressors is most frequently observed at the biochemical and physiological levels, such as those assessed for WoE in the section on *Physiological and biochemical effects in vivo*.

In the case of atrazine, there were a few studies on the ultimate-apical endpoint, the population. These studies and observations did not suggest adverse effects on fish or amphibians, and there were no studies on reptiles. However, studies at the level of the population are difficult to conduct because of the large number of uncontrolled variables, and are confounded by anthropogenic, biological, and physical stressors (see section on *Effects of atrazine at the level of the population*), many of which may mask the effects of lesser stressors.

In the absence of strong data at the population level, the measurement of the traditional apical endpoints for individuals (survival, development, reproduction, and growth) can be used. For atrazine, there were several apical endpoints, some with a large number of studies. Ideally, for use in the AOP framework, these studies should include measures of responses at lower levels of organization; however, these were rarely measured in a comprehensive way in the same study. The obverse of this, the inclusion of apical endpoints in studies on lower levels of organization was also rare, especially for responses such as immune function and behavior. However, with a sufficiently large number of studies, the responses can be generalized, even if they are from different studies and/or organisms.

Fortunately, atrazine is a well-studied chemical and a large number of publications did report apical endpoints, although these were not always associated with consistent measurements of responses lower on AOPs in the same study. To characterize these apical endpoints, we selected WoE assessments for percent hatching, short-term and long-term survival, successful development, weight or size, long-term survival, deformities, production of eggs, sex ratios, secondary sexual characteristics, smoltification of fish and survival of the salt-water challenge, and combined the mean scores in a WoE graphic (Figure 37). Overall, these apical endpoints had mean scores indicative of low relevance (<0.5) and a SOM of about 2, but could be relevant in an AOP. The obvious conclusion is that, at environmentally relevant concentrations, the risks of atrazine to fish, amphibians, and reptiles are *de minimis*. The one exception was that of survival of the SW challenge in *S. salar*, which had a mean relevance of $1.11 \pm$ SE 0.53 (see section on *Effects of atrazine on smoltification of fish…*); however, the variance on the mean score was greater than the other apical endpoints, indicating greater uncertainty and less concordance in the findings. This uncertainty was driven by very relevant effects on the survival of *S. salar* tested in one location (UK)

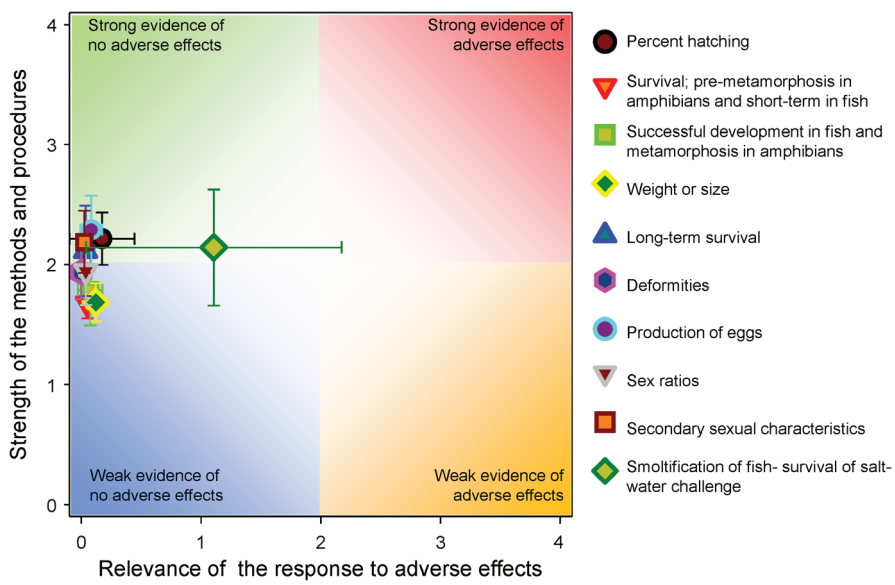

Figure 37. Overall summary of WoE assessments for apical endpoints. Symbols are the mean and the horizontal and vertical bars indicate $2 \times$ SE.

compared to others (US and Canada). Whether this is due to differences between populations of *S. salar* or the conditions of testing, is not clear. Even the results of studies from within the UK were highly varied.

Given the *de minimis* responses of apical endpoints, the analysis of AOPs is not informative. However, a major focus of the potential effects of atrazine on aquatic organisms has been on reproduction. Despite the lack of effects on the apical endpoint of reproduction itself, it is illustrative to assess the WoE on the AOP for reproduction. The scores for relevance and strength (Table 4) for moderately strong studies show evidence of no adverse effects on reproduction at all levels of organization of the AOP (with the exception of time to hatching, which was based on two studies only). The AOP is thus incomplete.

The separation of the analysis of WoE into relevance of the results and strength of the experimental procedures is useful as it allows uncertainty (SEs on the scores) to be identified. If this uncertainty is driven by weaknesses in methods, the information can be used to refine and improve experimental design. The WoE assessment did not include a formal analysis of risks; however, this was built into the scores in terms of characterization of the realism of concentrations used in the studies. If, as was not the case here, the WoE analysis identifies relevant responses, then a formal risk assessment can be conducted.

Common weaknesses in studies are discussed in the section on Methods, but one of the most important was lack of transparency of data in studies published in the literature. GLP studies include all of the raw data in the final report, which allows clarification of questions regarding the analysis of the data. WoE analyses of published papers would be greatly improved if raw data were provided in the form of SI. As is the practice is most journals, SI is often made available electronically, but the formats are not standardized and accessibility is not always easy (i.e., the SI is not automatically attached to a downloaded PDF of the paper). All journals should follow the lead of others, such as Proceedings of the National Academy of Sciences, and offer easy access to the SI or as has been suggested, the raw data as well (Schreider et al. 2010).

Table 4. WoE scores for relevance and strength of responses on the AOP for reproduction.

AOP level	Response	Score ± SE	
		Relevance	Strength
Macro-molecular	Estrogenicity and androgenicity	0.00 ± 0.00	2.20 ± 0.00
	Aromatase	0.08 ± 0.07	1.96 ± 0.15
Cell	Gonadal cell types	0.19 ± 0.04	2.20 ± 0.11
	Testicular ovarian follicles	0.04 ± 0.03	2.38 ± 0.11
Organ	GSI	0.13 ± 0.08	2.29 ± 0.14
	Gross abnormalities in testes	0.10 ± 0.07	2.18 ± 0.20
	Gross abnormalities in ovaries	0.10 ± 0.07	2.14 ± 0.23
	Production of eggs	0.08 ± 0.05	2.29 ± 0.14
	Estradiol	0.00 ± 0.00	2.24 ± 0.15
	Testosterone	0.19 ± 0.08	2.17 ± 0.13
	11-KT	0.25 ± 0.15	2.28 ± 0.14
Organism	Sex ratio	0.04 ± 0.03	1.95 ± 0.14
	Percent hatch	0.17 ± 0.14	2.22 ± 0.11
	Time to hatch	0.00 ± 0.00	1.50 ± 0.30
	Time to metamorphosis	0.13 ± 0.08	1.73 ± 0.12
	Successful development	0.07 ± 0.05	1.79 ± 0.15
	Secondary sexual characteristics	0.03 ± 0.02	2.19 ± 0.13

Overall, the general conclusion from this extensive and detailed WoE analysis is that atrazine does not adversely affect fish, amphibians, and reptiles at concentrations that are present in surface waters. This conclusion is consistent with those of others (APVMA 2008, 2010, Mann et al. 2009, MDA 2010a, b, USEPA 2007a, 2012c), but the methods used are different and are, to the extent possible, more transparent. We have been as inclusive as possible and have only excluded studies, such as those on mixtures, where it is not possible to assign causality. This method of WoE analysis could be applied to other chemicals and to other stressors; however, the scoring system might need to be adapted, especially if the responses are not toxicological.

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Declaration of interest

This study was funded by Syngenta Crop Protection LLC. Syngenta Crop Protection LLC is the primary registrant, producer, and marketer of atrazine herbicide. Atrazine is also marketed under license by Agan Chemical Manufacturing, Drexel Chemical Company, and Oxon Italia. AH is an employee of Syngenta Crop Protection LLC. GVDK, MLH, WK, and KRS acted as consultants to Syngenta in the conduct of this study. Their affiliations are listed under the authorship. The data and opinions in the paper were not edited by scientists or legal counsel representing the registrant, and none of the authors have been or are now engaged as expert witness on behalf of the registrant or other licensees of atrazine. GVDK, MLH, WK, and KRS have conducted other research on atrazine, which is included in this review and some of which has been presented at USEPA Science Advisor Panels on behalf of the registrant. The literature review strategy, the literature review, the WoE process, and the conclusions drawn are transparently described in detail in the paper and the accompanying supplemental information (SI) and are the exclusive professional work product of the authors. The opinions expressed are not necessarily those of Syngenta Crop Protection LLC, the licensees listed above, or the employers of the authors. Mention of trade names or commercial products do not constitute endorsement or recommendation for use.

References

Allran JW, and Karasov WH. (2000). Effects of atrazine and nitrate on northern leopard frog (*Rana pipiens*) larvae exposed in the laboratory from posthatch through metamorphosis. Environ Toxicol Chem, 19, 2850–5.

Allran JW, and Karasov WH. (2001). Effects of atrazine on embryos, larvae, and adults of anuran amphibians. Environ Toxicol Chem, 20, 769–75.

Andrus JM, Winter D, Scanlan M, Sullivan S, Bollman W, Waggoner JB, et al. (2013). Seasonal synchronicity of algal assemblages in three Midwestern agricultural streams having varying concentrations of atrazine, nutrients, and sediment. Sci Total Environ, 458, 125–39.

Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Hornung MW, Johnson RD, et al. (2010). Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. Environ Toxicol Chem, 29, 730–41.

APVMA. (2008). Atrazine Final Review Report and Regulatory Decision. Canberra, Australia: Australian Pesticides &Veterinary Medicines Authority. No. (number not available). 49 p. http://www.apvma.gov.au/products/review/docs/atrazine_finalMay08.pdf

APVMA. (2010). Atrazine Toxicity: Analysis of Potential Modes of Action. Canberra, Australia: Australian Pesticides &Veterinary Medicines Authority. No. (number not available). 49 p. http://www.apvma.gov.au/products/review/docs/atrazine_moa_june_2010.pdf

ASTM. (1992). Standard guide for conducting the Frog Embryo Teratogenesis Assay - *Xenopus* (FETAX). E1439-91. In *Annual Book of ASTM Standards*, Vol 11.04. Philadelphia, PA, pp 1199–1209.

Bailey LA, Prueitt RL, and Rhomberg LR. (2012). Hypothesis-based weight-of-evidence evaluation of methanol as a human carcinogen. Reg Toxicol Pharmacol, 62, 278–91.

Baker JL, and Laflen JM. (1979). Runoff losses of surface-applied herbicides as affected by wheel tracks and incorporation. J Environ Qual, 8, 602–7.

Baker LA, and Campbell PA. (1980). Thioglycolate medium decreases resistance to bacterial infection in mice. Infect Immun, 27, 455–60.

Battaglin WA, Rice KC, Focazio MJ, Salmons S, Barry RX. (2009). The occurrence of glyphosate, atrazine, and other pesticides in vernal pools and adjacent streams in Washington, DC, Maryland, Iowa, and Wyoming, 2005–2006. Environ Monit Assess, 155, 281–307.

Baxter L, Brain R, Rodriguez-Gil JL, Hosmer A, Solomon K, and Hanson M. (2014). Response of the green alga *Oophila sp*., a salamander endosymbiont, to a PSII-inhibitor under laboratory conditions. Environ Toxicol Chem, 33, 1858–64.

Baxter LR, Moore DL, Sibley PK, Solomon KR, and Hanson ML. (2011). Atrazine does not affect algal biomass or snail populations in microcosm communities at environmentally relevant concentrations. Environ Toxicol Chem, 30, 1689–96.

Baxter LR, Sibley PK, Solomon KR, and Hanson ML. (2013). Interactions between atrazine and phosphorus in aquatic systems: Effects on phytoplankton and periphyton. Chemosphere, 90, 1069–76.

BCPC. (2003). The e-Pesticide Manual Farnham, Surrey, UK: British Crop Protection Council.

Beebee TJ, and Griffiths RA. (2005). The amphibian decline crisis: a watershed for conservation biology? Biol Conserv, 125, 271–85.

Beldomenico PM, Rey F, Prado WS, Villarreal JC, Munõz-de-Toro M, and Luque EH. (2007). *In ovum* exposure to pesticides increases the egg weight loss and decreases hatchlings weight of *Caiman latirostris* (Crocodylia: Alligatoridae). Ecotoxicol Environ Safety, 68, 246–51.

Birge WJ, Black JA, and Kuehne RA. (1980). Effects of organic compounds on amphibian reproduction. University of Kentucky, Water Resources Research Institute. No. 121. 39 p.

Bishop CA, Mahony NA, Struger J, Ng P, and Pettit KE. (1999). Anuran development, density and diversity in relation to agricultural activity in the Holland River watershed, Ontario, Canada (1990–1992). Environ Monit Assess, 57, 21–43.

Bishop CA, Ashpole SL, Edwards AM, van Aggelen G, and Elliott JE. (2010a). Hatching success and pesticide exposures in amphibians living in agricultural habitats of the South Okanagan Valley, British Columbia, Canada (2004–2006). Environ Toxicol Chem, 29, 1593–603.

Bishop CA, McDaniel TV, and De Solla SR. (2010b). Atrazine in the Environment and its Implications for Amphibians and Reptiles. In: Sparling DW, Linder G, Bishop CA, and Krest SK, eds. Ecotoxicology of Amphibians and Reptiles. pp. 225–259. SETAC/CRC Press, Boca Raton.

Bisson M, and Hontela A. (2002). Cytotoxic and endocrine-disrupting potential of atrazine, diazinon, endosulfan, and mancozeb in adrenocortical steroidogenic cells of rainbow trout exposed in vitro. Toxicol Appl Pharmacol, 180, 110–17.

Blahová J, Plhalová L, Hostovsky M, Divišová L, Dobšíková R, Ivana M, et al. (2013). Oxidative stress responses in zebrafish *Danio rerio* after subchronic exposure to atrazine. Food Chem Toxicol, 61, 82–5.

Blahová J, Modra H, Sevcikova M, Marsalek P, Zelnickova L, Skoric M, and Svobodova Z. (2014). Evaluation of biochemical, haematological, and histopathological responses and recovery ability of common carp (*Cyprinus carpio* L.) after acute exposure to atrazine herbicide. Biomed Res Int, 2014, 980–48.

Blazer VS, Iwanowicz LR, Henderson H, Mazik PM, Jenkins JA, Alvarez DA, and Young JA. (2012). Reproductive endocrine disruption in smallmouth bass (*Micropterus dolomieu*) in the Potomac River basin: Spatial and temporal comparisons of biological effects. Environ Monit Assess, 184, 4309–34.

Boone MD, and James SM. (2003). Interactions of an insecticide, herbicide, and natural stressors in amphibian community mesocosms. Ecol Appl, 13, 829–41.

Borgert CJ, Mihaich EM, Ortego LS, Bentley KS, Holmes CM, Levine SL, and Becker RA. (2011). Hypothesis-driven weight of evidence framework for evaluating data within the US EPA's Endocrine Disruptor Screening Program. Reg Toxicol Pharmacol, 61, 185–91.

Borgert CJ, Stuchal LD, Mihaich EM, Becker RA, Bentley KS, Brausch JM, et al. (2014). Relevance weighting of Tier 1 endocrine screening endpoints by rank order. Birth Defects Res B, 101, 90–113.

Brain RA, and Brooks BW. (2012). Considerations and Criteria for the Incorporation of Mechanistic Sublethal Endpoints into Environmental Risk Assessment for Biologically Active Compounds. In: Brooks BW, and Huggett DB, eds. Human Pharmaceuticals in the Environment. Current and Future Perspectives. Volume **4**. pp. 139–166. Springer, New York, NY.

Breton RL, Gilron G, Thompson R, Rodney S, and Teed S. (2009). A new quality assurance system for the evaluation of ecotoxicity studies submitted under the new substances notification regulations in Canada. Integr Environ Assess Manag, 5, 127–37.

Bridges C, Little E, Gardiner D, Petty J, and Huckins J. (2004). Assessing the toxicity and teratogenicity of pond water in north-central Minnesota to amphibians. Environ Sci Res Int, 11, 233–9.

Bringolf RB, Belden JB, and Summerfelt RC. (2004). Effects of atrazine on fathead minnow in a short-term reproduction assay. Environ Toxicol Chem, 23, 1019–25.

Brock TCM, Lahr J, and van den Brink PJ. (2000). Ecological risks of pesticides in freshwater ecosystems Part 1: Herbicides. Wageningen, The Netherlands: Alterra. No. 088. 124 p.

Brodeur JC, Svartz G, Perez-Coll CS, Marino DJ, and Herkovits J. (2009). Comparative susceptibility to atrazine of three developmental stages of *Rhinella arenarum* and influence on metamorphosis: non-monotonous acceleration of the time to climax and delayed tail resorption. Aquat Toxicol, 91, 161–70.

Brodeur JC, Sassone A, Hermida GN, and Codugnello N. (2013). Environmentally-relevant concentrations of atrazine induce non-monotonic acceleration of developmental rate and increased size at metamorphosis in *Rhinella arenarum* tadpoles. Ecotoxicol Environ Safety, 92, 10–7.

Brodkin MA, Madhoun H, Rameswaran M, and Itzicj V. (2007). Atrazine is an immune disruptor in adult northern leopard frogs (*Rana pipiens*). Environ Toxicol Chem, 26, 80–4.

Brun GL, MacDonald RM, Verge J, and Aube J. (2008). Long-term atmospheric deposition of current-use and banned pesticides in Atlantic Canada; 1980–2000. Chemosphere, 71, 314–27.

Bull JJ, Gutzke WHN, and Crews D. (1988). Sex reversal by estradiol in three reptilian orders. Gen Comp Endocrinol, 70, 425–8.

Canestrelli D, Zampiglia M, and Nascetti G. (2013). Widespread occurrence of *Batrachochytrium dendrobatidis* in contemporary and historical samples of the endangered *Bombina pachypus* along the Italian Peninsula. PLoS ONE, 8, e63349.

Carr JA, Gentles A, Smith EE, Goleman WL, Urquidi LJ, Thuett K, et al. (2003). Response of larval *Xenopus laevis* to atrazine: assessment of gonadal and laryngeal morphology. Environ Toxicol Chem, 22, 396–405.

Carr JA, and Norris DO. (2006). The Hypothalamus-Pituitary Axis. In: Norris DO, and Carr JA, eds. Endocrine Disruption: The Biological Basis for Health Effects in Wildlife and Humans. pp. 87–110. Oxford University Press, New York, NY, USA.

Caruso NM, and Lips KR. (2013). Truly enigmatic declines in terrestrial salamander populations in Great Smoky Mountains National Park. Div Distrib, 19, 38–48.

Cavas T. (2011). In vivo genotoxicity evaluation of atrazine and atrazine-based herbicide on fish *Carassius auratus* using the micronucleus test and the comet assay. Food Chem Toxicol, 49, 1431–5.

Cericato L, Neto JG, Fagundes M, Kreutz LC, Quevedo RM, Finco J, et al. (2008). Cortisol response to acute stress in jundia *Rhamdia quelen* acutely exposed to sub-lethal concentrations of agrichemicals. Comp Biochem Physiol C, 148, 281–6.

Cericato L, Neto JG, Kreutz LC, Quevedo RM, da Rosa JG, Koakoski G, et al. (2009). Responsiveness of the interrenal tissue of Jundia (*Rhamdia quelen*) to an in vivo ACTH test following acute exposure to sublethal concentrations of agrichemicals. Comp Biochem Physiol C, 149, 363–7.

Chang CY, and Witschi E. (1956). Genetic control and hormonal reversal of sex differentiation in *Xenopus*. Proc Soc Exp Biol Med, 93, 140–4.

Chen T-H, Gross JA, and Karasov WH. (2009). Chronic exposure to pentavalent arsenic of larval leopard frogs (*Rana pipiens*): bioaccumulation and reduced swimming performance. Ecotoxicol, 18, 587–93.

Choung CB, Hyne RV, Mann RM, Stevens MM, and Hose GC. (2011). Developmental toxicity of two common corn pesticides to the endangered southern bell frog (*Litoria raniformis*). Environ Pollut, 159, 2648–55.

Christin M-S, Gendron AD, Brousseau P, Ménard L, Marcogliese DJ, Cyr D, et al. (2003). Effects of agricultural pesticides on the immune system of *Rana pipiens* and on its resistance to parasitic infection. Environ Toxicol Chem, 22, 1127–33.

Christin MS, Ménard L, Giroux I, Marcogliese DJ, Ruby S, Cyr D, et al. (2013). Effects of agricultural pesticides on the health of *Rana pipiens* frogs sampled from the field. Environ Sci Res Int, 20, 601–11.

Coady KK, Murphy MB, Villeneuve DL, Hecker M, Jones PD, Carr JA, et al. (2004). Effects of atrazine on metamorphosis, growth, and gonadal development in the green frog (*Rana clamitans*). J Toxicol Environ Health A, 67, 941–57.

Coady KK, Murphy MB, Villeneuve DL, Hecker M, Carr JA, Solomon KR, et al. (2005). Effects of atrazine on metamorphosis, growth, laryngeal and gonadal development, aromatase activity, and plasma sex steroid concentrations in *Xenopus laevis*. Ecotoxicol Environ Saf, 62, 160–73.

Corvi M, Stanley K, Peterson T, Kent M, Feist S, La Du J, et al. (2012). Investigating the impact of chronic atrazine exposure on sexual development in zebrafish. Birth Defects Res B, 95, 276–88.

Cossarini-Dunier M. (1987). Effects of the pesticides atrazine and lindane and of manganese ions on cellular-immunity of carp, *Cyprinus carpio*. J Fish Biol, 31, 67–73.

Cossarini-Dunier M, Demael A, Riviere JL, Lepot D. (1988). Effects of oral doses of the herbicide atrazine on carp (*Cyprinus carpio*). Ambio, 17, 401–405.

Cossarini-Dunier M, Hattenberger AM. (1988). Effect of pesticides (atrazine and lindane) on the replication of spring viremia of carp virus *in vitro*. Ann Res Vet, 19, 209–11.

Crain DA, Guillette LJ, Rooney AA, Pickford DB. (1997). Alterations in steroidogenesis in alligators (*Alligator mississippiensis*) exposed naturally and experimentally to environmental contaminants. Environ Health Perspect, 105, 528–53.

Crain DA, Spiteri ID, Guillette LJ, Jr. (1999). The functional and structural observations of the neonatal reproductive system of alligators exposed *in ovo* to atrazine, 2,4-D, or estradiol. Toxicol Ind Health, 15, 180–5.

Dalton RL, Nussbaumer C, Pick FR, Boutin C. (2013). Comparing the sensitivity of geographically distinct *Lemna minor* populations to atrazine. Ecotoxicol Environ Safety, 22, 718–30.

Davidson C. (2004). Declining downwind: Amphibian population declines in California and historical pesticide use. Ecol Appl, 14, 1892–902.

de Bravo MIS, Medina J, Marcano S, Finol HJ, Boada-Sucre A. (2005). Effects of herbicide on the kidneys of two Venezuelan cultured fish: *Caquetaia kraussii* and *Colossoma macropomum* (Pisces: Ciclidae and Characeae). Rev Biol Trop, 53, 55–60.

de Solla SR, Martin PA, Fernie KJ, Park BJ, Mayne G. (2006). Effects of environmentally relevant concentrations of atrazine on gonadal development of snapping turtles (*Chelydra serpentina*). Environ Toxicol Chem, 25, 520–26.

de Solla SR, Martin PA, Mikoda P. (2011). Toxicity of pesticide and fertilizer mixtures simulating corn production to eggs of snapping turtles (*Chelydra serpentina*). Sci Total Environ, 409, 4306–11.

del Carmen Alvarez M, Fuiman LA. (2005). Environmental levels of atrazine and its degradation products impair survival skills and growth of red drum larvae. Aquat Toxicol, 74, 229–41.

DeSesso JM, Scialli AR, White TEK, Breckenridge CB. (2014). Multigeneration reproduction and male developmental toxicity studies on atrazine in rats. Birth Defects Res B Dev Reprod Toxicol, 101, 237–53.

Detenbeck NE, Hermanutz R, Allen K, and Swift MC. (1996). Fate and effects of the herbicide atrazine in flow-through wetland mesocosms. Environ Toxicol Chem, 15, 937–46.

Dionne E. (1992). Atrazine Technical – Chronic Toxicity to the Fathead Minnow (*Pimephales promelas*) During a Full Life-Cycle Exposure. Greensboro, NC: Ciba-Geigy Corp, Unpublished Report No. 92-7-4324. Prepared by Springborn Laboratories, Inc., Wareham, MA. (MRID No. 425471–03).

Dong X, Zhu L, Wang J, Xie H, Hou X, Jia W. (2009). Effects of atrazine on cytochrome P450 enzymes of zebrafish (*Danio rerio*). Chemosphere, 77, 404–12.

Dorizzi M, Richard-Mercier G, Desvages G, Pieau C. (1994). Masculinization of gonads by aromatase inhibitors in a turtle with temperature-dependent sex determination. Differentiation, 58, 1–8.

Dornelles M, Oliveira G. (2013). Effect of atrazine, glyphosate and quinclorac on biochemical parameters, lipid peroxidation and survival in bullfrog tadpole (*Lithobates catesbeianus*). Arch Environ Contam Toxicol, 66, 415–29.

Du Preez HH, Kunene N, Hanner R, Giesy JP, Hosmer AJ, Solomon KR, Van Der Kraak GJ. (2009). Population-specific incidence of testicular ovarian follicles in *Xenopus laevis* from South Africa: A potential issue in endocrine testing. Aquat Toxicol, 95, 10–16.

Du Preez LH, Solomon KR, Carr JA, Giesy JP, Gross TS, Kendall RJ, Smith EE, Van Der Kraak GJ, Weldon C. (2005a). Population structure of the African Clawed Frog (*Xenopus laevis*) in maize-growing areas with atrazine application versus non-maize-growing areas in South Africa. Afr J Herp, 54, 61–68.

Du Preez LH, Jansen van Rensburg PJ, Jooste AM, Carr JA, Giesy JP, Gross TS, Kendall RJ, Smith EE, Van Der Kraak G, Solomon KR. (2005b). Seasonal exposures to triazine and other pesticides in surface waters in the western Highveld corn-production region in South Africa. Environ Pollut, 135, 131–41.

Du Preez LH, Kunene N, Everson GJ, Carr JA, Giesy JP, Gross TS, Kendall RJ, Smith EE, Solomon KR, Van Der Kraak GJ. (2008). Reproduction, larval growth, and reproductive development in African clawed frogs, (*Xenopus laevis*) exposed to atrazine. Chemosphere, 71, 546–52.

Edwards WM, Shipitalo MJ, Lal R, Owens LB. (1997). Rapid changes in concentration of herbicides in corn field surface depressions. J Soil Water Conserv, 52, 227–31.

Egea-Serrano A, Relyea RA, Tejedo M, Torralva M. (2012). Understanding of the impact of chemicals on amphibians: a meta-analytic review. Ecol Evol, 2, 1382–97.

Everson G. (2006). Reproductive biology of the African clawed frog, *Xenopus laevis*: A histometric analysis [M.Sc. thesis]. Potchefstroom, NWP, South Africa: North-West University.

Exponent. (2009). Technical Memorandum. Focused Literature Review of Weight-of-Evidence Approaches. Bellevue, WA: Exponent, (Report prepared for Dr. Paul West Chair, Science Advisory Board for Contaminated Sites in British Columbia). 58 p.

Fischer-Scherl T, Veeser A, Hoffmann RW, Kuhnhauser C, Negele RD, Ewringmann T. (1991). Morphological effects of acute and chronic atrazine exposure in rainbow trout (*Oncorhynchus mykiss*). Arch Environ Contam Toxicol, 20, 454–61.

Foradori CD, Hinds LR, Quihuis AM, Lacagina AF, Breckinridge CB, Handa RJ. (2011). The differential effect of atrazine on luteinizing hormone release in adrenalectomized adult female Wistar rats. Biol Reprod, 85, 684–9.

Foradori CD, Coder PS, Tisdel M, Yi KD, Simpkins JW, Handa RJ, and Breckenridge CB. (2014). The effect of atrazine administered by gavage or in diet on the LH surge and reproductive performance in intact female Sprague-Dawley and Long Evans rats. Birth Defects Res B Dev Reprod Toxicol, 101, 262–75.

Forson DD, Storfer A. (2006). Atrazine increases *Ranavirus* susceptibility in the tiger salamander, *Ambystoma tigrinum*. Ecol Appl, 16, 2325–32.

Fortin MG, Couillard CM, Pellerin J, Lebeuf M. (2008). Effects of salinity on sublethal toxicity of atrazine to mummichog (*Fundulus heteroclitus*) larvae. Mar Environ Res, 65, 158–70.

Fraites MJ, Cooper RL, Buckalew A, Jayaraman S, Mills L, Laws SC. (2009). Characterization of the hypothalamic-pituitary-adrenal axis response to atrazine and metabolites in the female rat. Toxicol Sci, 112, 88–99.

Freeman JL, Rayburn AL. (2004). In vivo genotoxicity of atrazine to anuran larvae. Mutat. Res, 560, 69–78.

Freeman JL, Rayburn AL. (2005). Developmental impact of atrazine on metamorphing *Xenopus laevis* as revealed by nuclear analysis and morphology. Environ Toxicol Chem, 24, 1648–53.

Friedman L. (2000). Why vote-count reviews don't count. Soc Biol Psych, 49, 161–2.

Fu Y, Li M, Liu C, Qu J-P, Zhu W-J, Xing H-J, Xu S-W, Li S. (2013). Effect of atrazine and chlorpyrifos exposure on cytochromeP450 contents and enzyme activities in common carp gills. Ecotoxicol Environ Safety, 94, 28–36.

Gibble RE, Baer KN. (2011). Effects of atrazine, agricultural runoff, and selected effluents on antimicrobial activity of skin peptides in *Xenopus laevis*. Ecotoxicol Environ Safety, 74, 593–9.

Giddings JM, Anderson TA, Hall LW, Jr, Kendall RJ, Richards RP, Solomon KR, Williams WM. (2005). A Probabilistic Aquatic Ecological Risk Assessment of Atrazine in North American Surface Waters. 432 p. Pensacola, FL, USA: SETAC Press.

Gluth G, Hanke WA. (1985). Comparison of physiological changes in carp, *Cyprinus carpio*, induced by several pollutants at sublethal concentrations. I. The dependency on exposure time. Ecotoxicol Environ Safety, 9, 179–88.

Goulet BN, and Hontela A. (2003). Toxicity of cadmium, endosulfan, and atrazine in adrenal steroidogenic cells of two amphibian species, *Xenopus laevis* and *Rana catesbeiana*. Environ Toxicol Chem, 22, 2106–113.

Gower DJ, Aberra RK, Schwaller S, Largen MJ, Collen B, Spawls S, et al. (2013). Long-term data for endemic frog genera reveal potential conservation crisis in the Bale Mountains, Ethiopia. Oryx, 47, 59–69.

Griggs JL, and Belden LK. (2008). Effects of atrazine and metolachlor on the survivorship and infectivity of *Echinostoma trivolvis* trematode cercariae. Arch Environ Chem Toxicol, 54, 195–202.

Grim B, and Steeger T. (2008). Collaboration between monitoring authorities, regulatory authorities and test facilities on GLP principles provides confidence in data quality with an emphasis on sound science. GLP: an example of a focused effort that paid off. Ann Ist Super Sanita, 44, 359–62.

Grim KC, Wolfe M, Hawkins W, Johnson RD, and Wolf J. (2007). Intersex in Japanese medaka (*Oryzias latipes*) used as negative controls in toxicological bioassays: A review of 54 cases from 41 studies. Environ Toxicol Chem, 26, 1636–43.

Gross TS. (2001a). Determination of Potential Effects of 10 Day Neonatal Exposure of Atrazine on Histological and Hormonal Sex Determination in Incubated American Alligator (*Alligator mississippiensis*) Eggs. Gainesville, FL, USA: University of Florida, Wildlife Reproductive Toxicology Laboratory. No. Number Wildlife-NOVA98.02a. 66 p.

Gross TS. (2001b). Determination of Potential Effects of 10 day Neonatal Exposure of Atrazine on Histological and Hormonal Sex Determination in Incubated Red-eared Slider (*Psuedemys elegans*) Eggs. Gainesville, FL, USA: University of Florida, Wildlife Reproductive Toxicology Laboratory. No. Number Wildlife-NOVA98.02b. 66 p.

Gunderson MP, Veldhoen N, Skirrow RC, Macnab MK, Ding W, van Aggelen G, and Helbing CC. (2011). Effect of low dose exposure to the herbicide atrazine and its metabolite on cytochrome P450 aromatase and steroidogenic factor-1 mRNA levels in the brain of premetamorphic bullfrog tadpoles (*Rana catesbeiana*). Aquat Toxicol, 102, 31–8.

Halstead NT, McMahon TA, Johnson SA, Raffel TR, Romansic JM, Crumrine PW, and Rohr JR. (2014). Community ecology theory predicts the effects of agrochemical mixtures on aquatic biodiversity and ecosystem properties. Ecol Lett, 17, 932–41.

Hayes TB. (1998). Sex determination and primary sex differentiation in amphibians: genetic and developmental mechanisms. J Exp Zool, 281, 373–99.

Hayes TB, Collins A, Mendoza M, Noriega N, Stuart AA, and Vonk A. (2002). Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proc Nat Acad Sci USA, 99, 5476–80.

Hayes TB, Haston K, Tsui M, Hoang A, Haeffele C, and Vonk A. (2003). Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): Laboratory and field evidence. Environ Health Perspect, 111, 568–75.

Hayes TB, Stuart AA, Mendoza M, Collins A, Noriega N, Vonk A, et al. (2006a). Characterization of atrazine-induced gonadal malformations in African clawed frogs (*Xenopus laevis*) and comparisons with effects of an androgen antagonist (cyproterone acetate) and exogenous estrogen (17ß-estradiol): Support for the demasculinization/feminization hypothesis. Environ Health Perspect, 114, 134–41.

Hayes TB, Case P, Chui S, Chung D, Haefele C, Haston K, et al. (2006b). Pesticide mixtures, endocrine disruption, and amphibian declines: Are we underestimating the impact? Environ Health Perspect, 114, 40–50.

Hayes TB, Khoury V, Narayan A, Nazir M, Park A, Brown T, et al. (2010). Atrazine induces complete feminization and chemical castration in male African clawed frogs (*Xenopus laevis*). Proc Nat Acad Sci USA, 107, 4612–7.

Hayes TB. (2011). Atrazine Has Been Used Safely for 50 Years? In: Elliott J. E., Bishop C. A., and Morrissey C. A., eds. Wildlife Ecotoxicology: Forensic Approaches. Volume **3**. pp. 301–324. Springer Science + Business Media, New York, Dordrecht, Heidelberg, London.

Hayes TB, Beasley VR, de Solla S, Iguchi T, Ingraham H, Kestemont P, et al. (2011). Demasculinization and feminization of male gonads by atrazine: Consistent effects across vertebrate classes. J Steroid Biochem Mol Biol, 127, 64–73.

Hecker M, Giesy JP, Jones PD, Jooste AM, Carr JA, Solomon KR, et al. (2004). Plasma sex steroid concentrations and gonadal aromatase activities in African clawed frogs (*Xenopus laevis*) from the corn-growing region of South Africa. Environ Toxicol Chem, 23, 1996–2007.

Hecker M, Kim WJ, Park J-W, Murphy MB, Villeneuve D, Coady KK, et al. (2005). Plasma concentrations of estradiol and testosterone, gonadal aromatase activity, and ultrastructure of the testis in *Xenopus laevis* exposed to estradiol and atrazine. Aquat Toxicol, 72, 383–96.

Hecker M, Murphy MB, Coady KK, Villeneuve DL, Jones PD, Carr JA, et al. (2006). Terminology of gonadal anomalies in fish and amphibians resulting from chemical exposures. Rev Environ Contam Toxicol, 187, 103–32.

Herman D, Kaushik NK, and Solomon KR. (1986). Impact of atrazine on periphyton in freshwater enclosures and some ecological consequences. Can J Fish Aquat Sci, 43, 1917–25.

Hill AB. (1965). The environment and disease: association or causation? Proc R Soc Med, 58, 295–300.

Hogan NS, Duarte P, Wade MG, Lean DR, and Trudeau VL. (2008). Estrogenic exposure affects metamorphosis and alters sex ratios in the northern leopard frog (*Rana pipiens*): Identifying critically vulnerable periods of development. Gen Comp Endocrinol, 156, 515–23.

Houck A, and Sessions SK. (2006). Could atrazine affect the immune system of the frog, *Rana pipiens*? Bios, 77, 107–12.

Howe GE, Gillis R, and Mowbray RC. (1998). Effects of chemical synergy and larval stage on the toxicity of atrazine and alachlor to amphibian larvae. Environ Toxicol Chem, 17, 519–25.

Hussein SY, ElNasser MA, and Ahmed SM. (1996). Comparative studies on the effects of herbicide atrazine on freshwater fish *Oreochromis niloticus* and *Chrysichthyes auratus* at Assiut, Egypt. Bull Environ Contam Toxicol, 57, 503–10.

IPCS. (2002). Global Assessment of the State-of-the-Science of Endocrine Disruptors. Geneva, Switzerland: International Programme on Chemical Safety of the World Health Organization. No. WHO/PCS/EDC/02.2. 180 p. http://www.who.int/pcs

Iwanowicz LR, Blazer VS, Guy CP, Pinkney AE, Mullican JE, and Alvarez DA. (2009). Reproductive health of bass in the Potomac, U.S.A., drainage: part 1. Exploring the effects of proximity to wastewater treatment plant discharge. Environ Toxicol Chem, 28, 1072–83.

Jantawongsri K, Thammachoti P, Kitana J, Khonsue W, and Kitana N. (2013). Comparison herbicide utilization in paddy field alters immune response of the rice frog *Fejervarya limnocharis* living in agricultural area at Nan Province, Thailand. ESHM, 2013, 1–6.

Jin Y, Chen R, Liu W, and Fu Z. (2010a). Effect of endocrine disrupting chemicals on the transcription of genes related to the innate immune system in the early developmental stage of zebrafish (*Danio rerio*). Fish Shellfish Immunol, 28, 854–61.

Jin Y, Zhang X, Shu L, Chen L, Sun L, Qian H, et al. (2010b). Oxidative stress response and gene expression with atrazine exposure in adult female zebrafish (*Danio rerio*). Chemosphere, 78, 846–52.

Jin Y, Zhang X, Lu D, and Fu Z. (2012). Proteomic analysis of hepatic tissue in adult female zebrafish (*Danio rerio*) exposed to atrazine. Arch Environ Contam Toxicol, 62, 127–34.

Jobling S, Burn RW, Thorpe K, Williams R, and Tyler C. (2009). Statistical modeling suggests that anti-androgens in wastewater treatment works effluents are contributing causes of widespread sexual disruption in fish living in English rivers. Environ Health Perspect, 117, 797–802.

Johnson PTJ, Chase JM, Dosch KL, Hartson RB, Gross JA, Larson DJ, et al. (2007). Aquatic eutrophication promotes pathogenic infection in amphibians. Proc Nat Acad Sci USA, 104, 15781–6.

Jooste AM, Du Preez LH, Carr JA, Giesy JP, Gross TS, Kendall RJ, et al. (2005). Gonadal development of *Xenopus laevis* larvae exposed through larval development to atrazine in outdoor microcosms. Environ Sci Technol, 39, 5255–61.

Kadoum AM, and Mock DE. (1978). Herbicide and insecticide residues in tailwater pits: water and pit bottom soil from irrigated corn and sorghum fields. J Agric Food Chem, 26, 45–50.

Kazeto Y, Place AR, and Trant JM. (2004). Effects of endocrine disrupting chemicals on the expression of CYP19 genes in zebrafish (*Danio rerio*) juveniles. Aquat Toxicol, 69, 25–34.

Kerby JL, and Storfer A. (2009). Combined effects of atrazine and chlorpyrifos on susceptibility of the tiger salamander to *Ambystoma tigrinum* virus. Eco Health, 6, 91–8.

Kerney R, Kim E, Hangarter RP, Heiss AA, Bishop CD, and Hall BK. (2011). Intracellular invasion of green algae in a salamander host. Proc Nat Acad Sci USA, 108, 6497–502.

Kier LD, and Kirkland DJ. (2013). Review of genotoxicity studies of glyphosate and glyphosate-based formulations. Crit Rev Toxicol, 43, 283–315.

Kiesecker JM. (2002). Synergism between trematode infection and pesticide exposure: a link to amphibian limb deformities in nature? Proc Nat Acad Sci USA, 99, 9900–4.

Klimisch H-J, Andreae M, and Tillmann U. (1997). A systematic approach for evaluating the quality of experimental toxicological and ecotoxicological data. Reg Toxicol Pharmacol, 25, 1–5.

Kloas W, Lutz I, Springer T, Krueger H, Wolf J, Holden L, and Hosmer A. (2009a). Does atrazine influence larval development and sexual differentiation in *Xenopus laevis*? Toxicol Sci, 107, 376–84.

Kloas W, Lutz I, Urbatzka R, Springer T, Krueger H, Wolf J, et al. (2009b). Does atrazine affect larval development and sexual differentiation of South African clawed frogs? Ann NY Acad Sci, 1163, 437–40.

Knapp RA, Boiano DM, and Vredenburg VT. (2007). Removal of nonnative fish results in population expansion of a declining amphibian (mountain yellow-legged frog, *Rana muscosa*). Biol Conserv, 135, 11–20.

Knight LA, Christenson MK, Trease AJ, Davis PH, and Kolok AS. (2013). The spring runoff in Nebraska's Elkhorn River watershed and its impact on two sentinel organisms. Environ Toxicol Chem, 32, 1544–51.

Knutson MG, Sauer JR, Olsen DA, Mossman MJ, Hemesath LM, and Lannoo MJ. (1999). Effects of landscape composition and wetland fragmentation on frog and toad abundance and species richness in Iowa and Wisconsin, U.S.A. Conserv Biol, 13, 1437–46.

Knutson MG, Richardson WB, Reineke DM, Gray BR, Parmelee JR, and Weick SE. (2004). Agricultural ponds support amphibian populations. Ecol Appl, 14, 669–84.

Koakoski G, Quevedo RM, Ferreira D, Oliveira TA, da Rosa JGS, de Abreu MS, et al. (2014). Agrichemicals chronically inhibit the cortisol response to stress in fish. Chemosphere, 112, 85–91.

Koch R. (1942). The aetiology of tuberculosis (translation of Die Aetiologie der Tuberculose [1882]). In: Clark DH, ed. Source book of medical history. pp. 392–406. Dover Publications, Inc., New York, NY, USA.

Kolpin DW, Sneck-Fahrer D, Hallberg GR, and Libra RD. (1997). Temporal trends of selected agricultural chemicals in Iowa's groundwater, 1982–1995: Are things getting better? J Environ Qual, 26, 1007–17.

Kolpin DW, Blazer VS, Gray JL, Focazio MJ, Young JA, Alvarez DA, et al. (2012). Chemical contaminants in water and sediment near fish nesting sites in the Potomac River basin: Determining potential exposures to smallmouth bass (*Micropterus dolomieu*). Sci. Tot. Environ, 443C, 700–16.

Koprivnikar J, Forbes MR, and Baker RL. (2006). Effects of atrazine on cercarial longevity, activity, and infectivity. J Parasitol, 92, 306–11.

Koprivnikar J, Forbes MR, and Baker RL. (2007). Contaminant effects on host–parasite interactions: Atrazine, frogs, and trematodes. Environ Toxicol Chem, 26, 2166–70.

Koprivnikar J. (2010). Interactions of environmental stressors impact survival and development of parasitized larval amphibians. Ecol Appl, 20, 2263–72.

Koprivnikar J, and Walker PA. (2011). Effects of the herbicide atrazine's metabolites on host snail mortality and production of trematode cercariae. J Parasitol, 97, 822–7.

Koprivnikar J, and Redfern JC. (2012). Agricultural effects on amphibian parasitism: importance of general habitat perturbations and parasite life cycles. J Wildl Dis, 48, 925–36.

Kreutz LC, Barcellos LJ, Marteninghe A, Dos Santos ED, and Zanatta R. (2010). Exposure to sublethal concentration of glyphosate or atrazine-based herbicides alters the phagocytic function and increases the susceptibility of silver catfish fingerlings (*Rhamdia quelen*) to *Aeromonas hydrophila* challenge. Fish Shellfish Immunol, 29, 694–7.

Kreutz LC, Barcellos LJ, Dos Santos ED, Pivato M, and Zanatta R. (2012). Innate immune response of silver catfish (*Rhamdia quelen*) exposed to atrazine. Fish Shellfish Immunol, 33, 1055–9.

Kreutz LC, Barcellos LJ, Dos Santos ED, Pivato M, and Zanatta R. (2013). Corrigendum to "Innate immune response of silver catfish (*Rhamdia quelen*) exposed to atrazine' [Fish Shellfish Immunol 33 (2012) 1055–1059]. Fish Shellfish Immunol, 35, 2051.

Krimsky S. (2005). The weight of scientific evidence in policy and law. Am J Pub Health, 95, S129–36.

Kroon FJ, Hook SE, Jones D, Metcalfe S, and Osborn HL. (2014). Effects of atrazine on endocrinology and physiology in juvenile barramundi, *Lates calcarifer* (Bloch). Environ Toxicol Chem, 33, 1607–14.

Kurt-Karakus PB, Teixeira C, Small J, Muir D, and Bidleman TF. (2011). Current-use pesticides in inland lake waters, precipitation, and air from Ontario, Canada. Environ Toxicol Chem, 30, 1539–48.

LaFiandra EM, Babbitt KJ, and Sower SA. (2008). Effects of atrazine on anuran development are altered by the presence of a nonlethal predator. J Toxicol Environ Health.A, 71, 505–11.

Langerveld AJ, Celestine R, Zaya R, Mihalko D, and Ide CF. (2009). Chronic exposure to high levels of atrazine alters expression of genes that regulate immune and growth-related functions in developing *Xenopus laevis* tadpoles. Environ Res, 109, 379–89.

Langlois VS, Carew AC, Pauli BD, Wade MG, Cooke GM, and Trudeau VL. (2010). Low levels of the herbicide atrazine alters sex ratios and reduces metamorphic success in *Rana pipiens* tadpoles raised in outdoor mesocosms. Environ Health Perspect, 118, 552–7.

Larson DL, McDonald S, Fivizzani AJ, Newton WE, and Hamilton SJ. (1998). Effect of the herbicide atrazine on *Ambystoma tigrinum* metamorphosis: Duration, larval growth and hormonal response. Physiol Zool, 71, 671–9.

Lawler SP, Dritz D, Strange T, and Holyoak M. (1999). Effects of introduced mosquitofish and bullfrogs on the threatened California red-legged frog. Conserv Biol, 13, 613–22.

Laws SC, Hotchkiss M, Ferrell J, Jayaraman S, Mills L, Modic W, et al. (2009). Chlorotriazine herbicides and metabolites activate an ACTH-dependent release of corticosterone in male Wistar rats. Toxicol Sci, 112, 78–87.

Le Mer C, Roy RL, Pellerin J, Couillard CM, and Maltais D. (2013). Effects of chronic exposures to the herbicides atrazine and glyphosate to larvae of the threespine stickleback (*Gasterosteus aculeatus*). Ecotoxicol Environ Safety, 89, 174–81.

LeBlanc GA, and Wang G. (2006). Chemical mixtures: Greater-than-additive effects? Environ Health Perspect, 114, A517–8.

Lenkowski JR, Reed JM, Deininger L, and McLaughlin KA. (2008). Perturbation of organogenesis by the herbicide atrazine in the amphibian *Xenopus laevis*. Environ Health Perspect, 116, 223–30.

Lenkowski JR, Sanchez-Bravo G, and McLaughlin KA. (2010). Low concentrations of atrazine, glyphosate, 2,4-dichlorophenoxyacetic acid, and triadimefon exposures have diverse effects on *Xenopus laevis* organ morphogenesis. J Environ Sci (China), 22, 1305–8.

Linkov I, Loney D, Cormier S, Satterstrom FK, and Bridges T. (2009). Weight-of-evidence evaluation in environmental assessment: review of qualitative and quantitative approaches. Sci Total Environ, 407, 5199–205.

Linkov I, Welle P, Loney D, Tkachuk A, Canis L, Kim JB, and Bridges T. (2011). Use of multicriteria decision analysis to support weight of evidence evaluation. Risk Anal, 31, 1211–25.

Liu XM, Shao JZ, Xiang LX, and Chen XY. (2006). Cytotoxic effects and apoptosis induction of atrazine in a grass carp (*Ctenopharyngodon idellus*) cell line. Environ Toxicol, 21, 80–9.

Lynch TR, Johnson HE, and Adams WJ. (1985). Impact of atrazine and hexachlorobiphenyl on the structure and function of model stream ecosystems. Environ Toxicol Chem, 4, 399–413.

Macek KJ, Buxton KS, Sauter S, Gnilka S, and Dean JW. (1976). Chronic Toxicity of Atrazine to Selected Aquatic Invertebrates and Fishes. Duluth, MN: US Environmental Protection Agency, Office of Research and Development, Environmental Research Laboratory. No. EPA-600/3-76-047 (MRID # 000243-77). 49 p.

Mann RM, Hyne RV, Choung CB, and Wilson SP. (2009). Amphibians and agricultural in a complex environment. Environ Pollut, 157, 2907–27.

Marco A, and Blaustein AR. (2000). Symbiosis with green algae affects survival and growth of Northwestern salamander embryos. J Herpetol, 34, 617–21.

Marcogliese DJ, and Pietrock M. (2010). Combined effects of parasites andcontaminants on animal health: Parasites do matter. Trend Parasitol, 27, 123–30.

Martyniuk CJ, Sanchez BC, Szabo NJ, Denslow ND, and Sepulveda MS. (2009). Aquatic contaminants alter genes involved in neurotransmitter synthesis and gonadotropin release in largemouth bass. Aquat Toxicol, 95, 1–9.

Matsumoto J, Hosmer AJ, and Van Der Kraak G. (2010). Survival and iono-regulatory performance in Atlantic salmon smolts is not affected by atrazine exposure. Comp Biochem Physiol C, 152, 379–84.

McCarthy ID, and Fuiman LA. (2008). Growth and protein metabolism in red drum (*Sciaenops ocellatus*) larvae exposed to environmental levels of atrazine and malathion. Aquat Toxicol, 88, 220–9.

McCarty LS, Borgert CJ, and Mihaich EM. (2012). Information quality in regulatory decision-making: Peer review versus good laboratory practice. Environ Health Perspect, 120, 927–34.

McCoy KA, Bortnick LJ, Campbell CM, Hamlin HJ, Guillette LJ, Jr, and St. Mary CM. (2008). Agriculture alters gonadal form and function in the toad *Bufo marinus*. Environ. Health Perspect, 116, 1526–32.

McDaniel T, Martin PA, Struger J, Sherry J, Marvin CH, McMaster ME, et al. (2008). Potential endocrine disruption of sexual development in free ranging male northern leopard frogs (*Rana pipiens*) and green frogs (*Rana clamitans*) from areas of intensive row crop agriculture. Aquat Toxicol, 88, 230–42.

McDonald BG, deBruyn AMH, Wernick BG, Patterson L, Pellerin N, and Chapman PM. (2007). Design and application of a transparent and scalable weight-of-evidence framework: an example from Wabamun Lake, Alberta, Canada. Integr Environ Assess Manag, 4, 476–83.

McMahon TA, Romansic JM, and Rohr JR. (2013). Nonmonotonic and monotonic effects of pesticides on the pathogenic fungus *Batrachochytrium dendrobatidis* in culture and on tadpoles. Environ Sci Technol, 47, 7958–64.

MDA. (2010a). Summary of the Special Registration Review of the Herbicide Atrazine in Minnesota. Minneapolis, MN, USA: Minnesota Department of Agriculture. Special Registration Review. No. Number not available. 13 p. http://www.mda.state.mn.us/chemicals/pesticides/atrazine/~/media/Files/chemicals/atrazine/reviewsummary.ashx

MDA. (2010b). Atrazine Technical Assessments. Minneapolis, MN, USA: Minnesota Department of Agriculture. Special Registration Review. No. Number not available. 92 p. http://www.mda.state.mn.us/chemicals/pesticides/atrazine/~/media/Files/chemicals/atrazine/techassessments.ashx

Mehler WT, Schuler LJ, and Lydy MJ. (2008). Examining the joint toxicity of chlorpyrifos and atrazine in the aquatic species: *Lepomis macrochirus*, *Pimephales promelas* and *Chironomus tentans*. Environ Pollut, 152, 217–24.

Mela M, Guiloski IC, Doria HB, Randi MAF, deOliveira Ribeiro CA, Pereira L, et al. (2013). Effects of the herbicide atrazine in neotropical catfish (*Rhamdia quelen*). Ecotoxicol Environ Safety, 93, 13–21.

Mitchkash MG, McPeek T, and Boone MD. (2014). The effects of 24-h exposure to carbaryl or atrazine on the locomotor performance and overwinter growth and survival of juvenile spotted salamanders (*Ambystoma maculatum*). Environ Toxicol Chem, 33, 548–52.

Modic MW. (2004). The role of testicular aromatase in the atrazine mediated changes of estrone and estradiol in the male wistar rat [Ph.D. thesis]: North Carolina State University.

Moore A, and Waring CP. (1998). Mechanistic effects of a triazine pesticide on reproductive endocrine function in mature male Atlantic salmon (*Salmo salar* L.) parr. Pestic Biochem Physiol, 62, 41–50.

Moore A, and Lower N. (2001). The impact of two pesticides on olfactory-mediated endocrine function in mature male Atlantic salmon (*Salmo salar* L.) parr. Comp Biochem Physiol B, 129, 269–76.

Moore A, Scott AP, Lower N, Katsiadaki I, and Greenwood L. (2003). The effect of 4-nonylphenol and atrazine on Atlantic salmon (*Salmo salar* L.) smolts. Aquacult, 222, 253–63.

Moore A, Lower N, Mayer I, and Greenwood L. (2007). The impact of a pesticide on migratory activity and olfactory function in Atlantic salmon (*Salmo salar* L.) smolts. Aquacult, 273, 350–9.

Moore A, Cotter D, Quayle V, Rogan G, Poole R, Lower N, and Privitera L. (2008). The impact of a pesticide on the physiology and behaviour of hatchery-reared Atlantic salmon, *Salmo salar*, smolts during the transition from fresh water to the marine environment. Fish Manage Ecol, 15, 385–92.

Morgan MK, Scheuerman PR, Bishop CS, and Pyles RA. (1996). Teratogenic potential of atrazine and 2,4-D using FETAX. J Toxicol Environ Health, 48, 151–68.

Muncke J, Junghans M, and Eggen RI. (2007). Testing estrogenicity of known and novel (xeno-)estrogens in the MolDarT using developing zebrafish (*Danio rerio*). Environ Toxicol, 22, 185–93.

Murphy MB, Hecker M, Coady KK, Tompsett AR, Du Preez LH, Everson GJ, et al. (2006a). Atrazine concentrations, gonadal gross morphology and histology in ranid frogs collected in Michigan agricultural areas. Aquat Toxicol, 76, 230–45.

Murphy MB, Hecker M, Coady KK, Tompsett AR, Higley EB, Jones PD, et al. (2006b). Plasma steroid hormone concentrations, aromatase activities and GSI in ranid frogs collected from agricultural and non-agricultural sites in Michigan (USA). Aquat Toxicol, 77, 153–66.

Nadzialek S, Spano L, Mandiki SN, and Kestemont P. (2008). High doses of atrazine do not disrupt activity and expression of aromatase in female gonads of juvenile goldfish (*Carassius auratus* L.). Ecotoxicology, 17, 464–70.

Nascimento CR, Souza MM, and Martinez CB. (2012). Copper and the herbicide atrazine impair the stress response of the freshwater fish *Prochilodus lineatus*. Comp Biochem Physiol C, 155, 456–61.

Nature. (2010). The weight of evidence (editorial). Nature, 464, 1103–4.

Neuman-Lee LA, and Janzen FJ. (2011). Atrazine exposure impacts behavior and survivorship of neonatal turtles. Herpetologica, 67, 23–31.

Neuman-Lee LA, Gaines KF, Baumgartner KA, Voorhees JR, Novak JM, and Mullin SJ. (2013). Assessing multiple endpoints of atrazine ingestion on gravid northern watersnakes (*Nerodia sipedon*) and their offspring. Environ Toxicol, doi:,10.1002/tox.21837.

Nieves-Puigdoller K, Björnsson BT, and McCormick SD. (2007). Effects of hexazinone and atrazine on the physiology and endocrinology of smolt development in Atlantic salmon. Aquat Toxicol, 84, 27–37.

Nwani CD, Lakra WS, Nagpure NS, Kumar R, Kushwaha B, and Srivastava SK. (2010). Toxicity of the herbicide atrazine: Effects on lipid peroxidation and activities of antioxidant enzymes in the freshwater fish *Channa punctatus* (Bloch). Int J Environ Res Pub Health, 7, 3298–312.

Nwani CD, Nagpure NS, Kumar R, Kushwaha B, Kumar P, and Lakra WS. (2011). Mutagenic and genotoxic assessment of atrazine-based herbicide to freshwater fish *Channa punctatus* (Bloch) using micronucleus test and single cell gel electrophoresis. Environ Toxicol Pharmacol, 31, 314–22.

OECD. (2012). Test No. 229: Fish Short Term Reproduction Assay, OECD Guidelines for the Testing of Chemicals, Section 2: Effects on Biotic Systems. Paris, France: OECD. 40 p. http://www.oecd-ilibrary.org/environment/test-no-229-fish-short-term-reproduction-assay_9789264185265-en;jsessionid = 6te0o79apq233.x-oecd-live-01

Oka T, Tooi O, Mitsui N, Miyahara M, Ohnishi Y, Takase M, et al. (2008). Effect of atrazine on metamorphosis and sexual differentiation in *Xenopus laevis*. Aquat Toxicol, 87, 215–26.

Olivier HM, and Moon BR. (2010). The effects of atrazine on spotted salamander embryos and their symbiotic alga. Ecotoxicology, 19, 654–61.

Olmstead AW, Kosian PA, Korte JJ, Holcombe GW, Woodis KK, and Degitz SJ. (2009). Sex reversal of the amphibian, *Xenopus tropicalis*, following larval exposure to an aromatase inhibitor. Aquat Toxicol, 91, 143–50.

Olson DH, Aanensen DM, Ronnenberg KL, Powell CI, Walker SF, Bielby J, et al. (2013). Mapping the global emergence of

*Batrachochytrium dendrobatidi*s, the amphibian chytrid fungus. PLoS ONE, 8, e56802.
Orton F, Carr JA, and Handy RD. (2006). Effects of nitrate and atrazine on larval development and sexual differentiation in the Northern Leopard frog *Rana pipiens*. Environ Toxicol Chem, 25, 65–71.
Orton F, Lutz I, Kloas W, and Routledge EJ. (2009). Endocrine disrupting effects of herbicides and pentachlorophenol: *In vitro* and *in vivo* evidence. Environ Sci Technol, 43, 2144–50.
Oulmi Y, Negele RD, and Braunbeck T. (1995). Segment specificity of the cytological response in rainbow trout (*Oncorhynchus mykiss*) renal tubules following prolonged exposure to sublethal concentrations of atrazine. Ecotoxicol Environ Safety, 32, 39–50.
Paetow LJ, Daniel McLaughlin J, Cue RI, Pauli BD, and Marcogliese DJ. (2012). Effects of herbicides and the chytrid fungus *Batrachochytrium dendrobatidis* on the health of post-metamorphic northern leopard frogs (*Lithobates pipiens*). Ecotoxicol Environ Safety, 80, 372–80.
Pandian TJ, and Sheela SG. (1995). Hormonal induction of sex reversal in fish. Aquacult, 138, 1–22.
Papoulias DM, Schwarz MS, and Mena L. (2013). Gonadal abnormalities in frogs (*Lithobates spp*.) collected from managed wetlands in an agricultural region of Nebraska, USA. Environ Pollut, 172, 1–8.
Papoulias DM, Tillitt DE, Talykina MG, Whyte JJ, and Richter CA. (2014). Atrazine reduces reproduction in Japanese medaka (*Oryzias latipes*). Aquat Toxicol, 154, 230–9.
Paulino MG, Souza NE, and Fernandes MN. (2012a). Subchronic exposure to atrazine induces biochemical and histopathological changes in the gills of a Neotropical freshwater fish, *Prochilodus lineatus*. Ecotoxicol Environ Safety, 80, 6–13.
Paulino MG, Sakuragui MM, and Fernandes MN. (2012b). Effects of atrazine on the gill cells and ionic balance in a neotropical fish, *Prochilodus lineatus*. Chemosphere, 86, 1–7.
Pieau C, Dorizzi M, and Richard-Mercier N. (1999). Temperature-dependent sex determination and gonadal differentiation in reptiles. Cell Mol Life Sci, 55, 887–900.
Plhalova L, Blahova J, Mikulikova I, Stepanova S, Dolezelova P, Praskova E, et al. (2012). Effects of subchronic exposure to atrazine on zebrafish (*Danio rerio*). Pol J Vet Sci, 15, 417–23.
Polakiewicz FJ, and Goodman RM. (2013). The effects of environmental stressors and the pathogen Ranavirus on survival and health of juvenile freshwater turtles. Hampden-Sydney College J Sci, 2, 1–6.
Prasad TA, and Reddy DC. (1994). Atrazine toxicity on hydromineral balance of fish, *Tilapia mossambicus*. Ecotoxicol Environ Safety, 28, 313–6.
Prueitt RL, Goodman JE, Bailey LA, and Rhomberg LR. (2011). Hypothesis-based weight-of-evidence evaluation of the neurodevelopmental effects of chlorpyrifos. Crit Rev Toxicol, 41, 822–903.
Pruett SB, Fan R, Zheng Q, Myers LP, and Hebert P. (2003). Modeling and predicting immunological effects of chemical stressors: Characterization of a quantitative biomarker for immunological changes caused by atrazine and ethanol. Toxicol Sci, 75, 343–54.
Pruett SB, Fan R, Zheng Q, and Schwab C. (2009). Patterns of immunotoxicity associated with chronic as compared to acute exposure to chemical or physical stressors and their relevance with regard to the role of stress and with regard to immunotoxicity testing. Toxicol Sci, 109, 265–75.
Ramsingh D. (2010). The Assessment of the Chronic Toxicity and Carcinogenicity of Pesticides. In: Krieger RI, Doull J, van Hemmen JJ, Hodgson E, Maibach HI, Ritter L, Ross J, and Slikker W, eds. Handbook of Pesticide Toxicology3 ed. pp. 463–482. Elsevier, Burlington, MA, USA.
Reeder AL, Foley GL, Nichols DK, Hansen LG, Wikoff B, Faeh S, et al. (1998). Forms and prevalence of intersexuality and effects of environmental contaminants on sexuality in cricket frogs (*Acris crepitans*). Environ Health Perspect, 106, 261–6.
Reeder AL, Ruiz MO, Pessier A, Brown LE, Levengood JM, Phillips CA, et al. (2005). Intersexuality and the cricket frog decline: Historic and geographic trends. Environ Health Perspect, 113, 261–5.
Reeder NMM, Pessier AP, and Vredenburg VT. (2012). A reservoir species for the emerging amphibian pathogen *Batrachochytrium dendrobatidis* thrives in a landscape decimated by disease *PLoS ONE.* 7, e33567.
Reid N, Dingerkus SK, Stone RE, Pietravalle S, Kelly R, Buckley J, et al. (2013). Population enumeration and assessing conservation status in a widespread amphibian: a case study of *Rana temporaria* in Ireland. Animal Conserv, 16, 519–27.
Relyea RA. (2009). A cocktail of contaminants: how mixtures of pesticides at low concentrations affect aquatic communities. Oecologia, 159, 363–376.
Rey F, Gonzalez M, Zayas MA, Stoker C, Durando M, Luque EH, and Munoz-de-Toro M. (2009). Prenatal exposure to pesticides disrupts testicular histoarchitecture and alters testosterone levels in male *Caiman latirostris*. Gen Comp Endocrinol, 162, 286–92.
Rhen T, and Lang JW. (1994). Temperature-dependent sex determination in the snapping turtle: Manipulation of the embryonic sex steroid environment. Gen Comp Endocrinol, 96, 243–54.
Rhomberg LR, Bailey LA, and Goodman JE. (2010). Hypothesis-based weight of evidence: a tool for evaluating and communicating uncertainties and inconsistencies in the large body of evidence in proposing a carcinogenic mode of action–naphthalene as an example. Crit Rev Toxicol, 40, 671–96.
Rhomberg LR, Bailey LA, Goodman JE, Hamade AK, and Mayfield D. (2011). Is exposure to formaldehyde in air causally associated with leukemia?–A hypothesis-based weight-of-evidence analysis. Crit Rev Toxicol, 41, 555–621.
Rhomberg LR, Goodman JE, Foster WG, Borgert CJ, and Van Der Kraak G. (2012). A critique of the European Commission Document, 'State of the Art Assessment of Endocrine Disrupters". Crit Rev Toxicol, 42, 465–73.
Rider CV, Hartig PC, Cardon MC, Lambright CR, Bobseine KL, Guillette LJ, Jr., et al. (2010). Differences in sensitivity but not selectivity of xenoestrogen binding to alligator versus human estrogen receptor alpha. Environ Toxicol Chem, 29, 2064–71.
Rohr JR, Elskus AA, Shepherd BS, Crowley PH, McCarthy TM, Neidzwiecki JH, et al. (2003). Lethal and sublethal effects of atrazine, carbaryl, endosulfan, and octylphenol on the streamside salamander (*Ambystoma barbouri*). Environ Toxicol Chem, 22, 2385–92.
Rohr JR, Elskus AA, Shepherd BS, Crowley PH, McCarthy TM, Niedzwiecki JH, et al. (2004). Multiple stressors and salamanders: Effects of an herbicide, food limitation, and hydroperiod. Ecol Appl, 14, 1028–40.
Rohr JR, and Crumrine PW. (2005). Effects of an herbicide and an insecticide on pond community structure and processes. Ecol Appl, 15, 1135–47.
Rohr JR, and Palmer BD. (2005). Aquatic herbicide exposure increases salamander desiccation risk eight months later in a terrestrial environment. Environ Toxicol Chem, 24, 1253–8.
Rohr JR, Sager T, Sesterhenn TM, and Palmer BD. (2006). Exposure, postexposure, and density-mediated effects of atrazine on amphibians: Breaking down net effects into their parts. Environ Health Perspect, 114, 46–50.
Rohr JR, Schotthoefer AM, Raffel TR, Carrick HJ, Halstead N, Hoverman JT, et al. (2008a). Agrochemicals increase trematode infections in a declining amphibian species. Nature, 455, 1235–9.
Rohr JR, Raffel TR, Sessions SK, and Hudson PJ. (2008b). Understanding the net effects of pesticides on amphibian trematode infections. Ecol Appl, 18, 1743–53.
Rohr JR, Swan A, Raffel TR, and Hudson PJ. (2009). Parasites, info-disruption, and the ecology of fear. Oecologia, 159, 447–54.
Rohr JR, and McCoy KA. (2010a). A qualitative meta-analysis reveals consistent effects of atrazine on freshwater fish and amphibians. Environ Health Perspect, 118, 20–32.
Rohr JR, and McCoy KA. (2010b). Preserving environmental health and scientific credibility: A practical guide to reducing conflicts of interest. Conserv. Lett, 3, 143–50.
Rohr JR, and Raffel TR. (2010). Linking global climate and temperature variability to widespread amphibian declines putatively caused by disease. Proc Nat Acad Sci USA, 107, 8269–74.
Rohr JR, Sesterhenn TM, and Stieha C. (2011). Will climate change reduce the effects of a pesticide on amphibians?: Partitioning effects on exposure and susceptibility to contaminants. Glob Change Biol, 17, 657–66.
Rohr JR, Halstead NT, and Raffel TR. (2012). The herbicide atrazine, algae, and snail populations. Environ Toxicol Chem, 31, 973–4; author reply 974–6.
Rohr JR, and Palmer BD. (2013). Climate change, multiple stressors, and the decline of ectotherms. Conserv Biol, 27, 741–51.
Rohr JR, Raffel TR, Halstead NT, McMahon TA, Johnson SA, Boughton RK, and Martin LB. (2013). Early-life exposure to a herbicide has

enduring effects on pathogen-induced mortality. Proc R Soc B Biol Sci, 280, 20131502.
Saglio P, and Trijasse S. (1998). Behavioral responses to atrazine and diuron in goldfish. Arch Environ Contam. Toxicol, 35, 484–91.
Salaberria I, Hansen BH, Asensio V, Olsvik PA, Andersen RA, and Jenssen BM. (2009). Effects of atrazine on hepatic metabolism and endocrine homeostasis in rainbow trout (*Oncorhynchus mykiss*). Toxicol Appl Pharmacol, 234, 98–106.
Sanchez BC, Ralston-Hooper KJ, Kowalski KA, Dorota Inerowicz H, Adamec J, and Sepulveda MS. (2009). Liver proteome response of largemouth bass (*Micropterus salmoides*) exposed to several environmental contaminants: potential insights into biomarker development. Aquat Toxicol, 95, 52–9.
Sanchez BC, Carter B, Hammers HR, and Sepulveda MS. (2011). Transcriptional response of hepatic largemouth bass (*Micropterus salmoides*) mRNA upon exposure to environmental contaminants. J Appl Toxicol, 31, 108–16.
Sanderson JT, Seinen W, Giesy JP, and van den Berg M. (2000). 2-Chloro-s-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity? Toxicol Sci, 54, 121–7.
Sanderson JT, Letcher RJ, Heneweer M, Giesy JP, and van den Berg M. (2001). Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitelllogenin production in male carp hepatocytes. Environ Health Perspect, 109, 1027–31.
Schmidel AJ, Assmann KL, Werlang CC, Bertoncello KT, Francescon F, Rambo CL, et al. (2014). Subchronic atrazine exposure changes defensive behaviour profile and disrupts brain acetylcholinesterase activity of zebrafish. Neurotoxicol Teratolog, 44, 62–9.
Schotthoefer AM. (2003). Environmental, landscape, and host-related factors associated with parasitism and its effects on larvae and metamorphic frogs of *Rana pipiens* [Ph.D. thesis]. Urbana, IL, USA: University of Illinois, 195 p.
Schreider J, Barrow C, Birchfield N, Dearfield K, Devlin D, Henry S, et al. (2010). Enhancing the credibility of decisions based on scientific conclusions: Transparency is imperative. Toxicol Sci, 116, 5–7.
Schwab CL, Fan R, Zheng Q, Myers LP, Hebert P, and Pruett SB. (2005). Modeling and predicting stress-induced immunosuppression in mice using blood parameters. Toxicol Sci, 83, 101–13.
Scialli AR, DeSesso JM, and Breckenridge CB. (2014). Developmental toxicity studies with atrazine and its major metabolites in rats and rabbits. Birth Defects Res B Dev Reprod Toxicol, 101, 199–214.
Shelley LK, Ross PS, and Kennedy CJ. (2012a). Immunotoxic and cytotoxic effects of atrazine, permethrin and piperonyl butoxide to rainbow trout following in vitro exposure. Fish Shellfish Immunol, 33, 455–8.
Shelley LK, Ross PS, Miller KM, Kaukinen KH, and Kennedy CJ. (2012b). Toxicity of atrazine and nonylphenol in juvenile rainbow trout (*Oncorhynchus mykiss*): Effects on general health, disease susceptibility and gene expression. Aquat. Toxicol, 124–125, 217–26.
Shenoy K. (2012). Environmentally realistic exposure to the herbicide atrazine alters some sexually selected traits in male guppies. PLoS ONE, 7, e30611.
Sifkarovski J, Grayfer L, De Jesús Andino F, Lawrence BP, and Robert J. (2014). Negative effects of low dose atrazine exposure on the development of effective immunity to FV3 in *Xenopus laevis*. Dev Comp Immunol, 47, 52–58.
Simpkins JW, Swenberg JS, Weiss N, Brusick D, Eldridge JC, Stevens JT, et al. (2011). Atrazine and breast cancer: A framework assessment of the toxicological and epidemiological evidence. Toxicol Sci, 123, 441–59.
Skelly DK, Bolden SR, and Dion KB. (2010). Intersex frogs concentrated in suburban and urban landscapes. Ecohealth, 7, 374–9.
Smith EE, du Preez LH, Gentles BA, Solomon KR, Tandler B, Carr JA, et al. (2005). Assessment of laryngeal muscle and testicular cell types in *Xenopus laevis* (Anura Pipidae) inhabiting maize and non-maize growing areas of South Africa. Afr J Herp, 54, 69–76.
Solomon KR, Baker DB, Richards P, Dixon KR, Klaine SJ, La Point TW, et al. (1996). Ecological risk assessment of atrazine in North American surface waters. Environ Toxicol Chem, 15, 31–76.
Solomon KR, Carr JA, Du Preez LH, Kendall RJ, Smith EE, and Van Der Kraak GJ. (2008). Effects of atrazine on fish, amphibians, and aquatic reptiles: a critical review. Crit Rev Toxicol, 38, 721–72.
Spanó L, Tyler CR, Van Aerle R, Devos P, Mandiki SNM, Thomé J-P, and Kestemont P. (2004). Effects of atrazine on sex steroid dynamics, plasma vitellogenin concentration and gonad development in adult goldfish (*Carasius auratus*). Aquat Toxicol, 66, 369–79.
Spolyarich N, Hyne R, Wilson S, Palmer C, and Byrne M. (2010). Growth, development and sex ratios of Spotted Marsh Frog (*Limnodynastes tasmaniensis*) larvae exposed to atrazine and a herbicide mixture. Chemosphere, 78, 807–13.
Spolyarich N, Hyne RV, Wilson SP, Palmer CG, and Byrne M. (2011). Morphological abnormalities in frogs from a rice-growing region in NSW, Australia, with investigations into pesticide exposure. Environ Monit Assess, 173, 397–407.
Stoker C, Beldomenico PM, Bosquiazzo VL, Zayas MA, Rey F, Rodriguez H, et al. (2008). Developmental exposure to endocrine disruptor chemicals alters follicular dynamics and steroid levels in *Caiman latirostris*. Gen Comp Endocrinol, 156, 603–12.
Storrs-Mendez SI, and Semlitsch RD. (2010). Intersex gonads in frogs: understanding the time course of natural development and role of endocrine disruptors. J Exp Zool B Mol Dev Evol, 314, 57–66.
Storrs Mendez SI, Tillitt DE, Rittenhouse TA, and Semlitsch RD. (2009). Behavioral response and kinetics of terrestrial atrazine exposure in American toads (*Bufo americanus*). Arch Environ Contam Toxicol, 57, 590–7.
Storrs SI, and Kiesecker JM. (2004). Survivorship patterns of larval amphibians exposed to low concentrations of atrazine. Environ. Health Perspect, 112, 1054–7.
Storrs SI, and Semlitsch RD. (2008). Variation in somatic and ovarian development: predicting susceptibility of amphibians to estrogenic contaminants. Gen. Comp. Endocrinol, 156, 524–30.
Suzawa M, and Ingraham HA. (2008). The herbicide atrazine activates endocrine gene networks via non-steroidal NR5A nuclear receptors in fish and mammalian cells. PLoS ONE, 3, e2117.
Tavera-Mendoza L, Ruby S, Brousseau P, Fourier M, Cyr D, and Marcogliese D. (2002a). Response of the amphibian tadpole *Xenopus laevis* to atrazine during sexual differentiation of the ovary. Environ Toxicol Chem, 21, 1264–7.
Tavera-Mendoza L, Ruby S, Brousseau P, Fourier M, Cyr D, and Marcogliese D. (2002b). Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the testis. Environ. Toxicol Chem, 21, 527–31.
Tavera-Mendoza LE. (2001). Influences of atrazine on gonadal differentiation in *Xenopus laevis* tadpoles during metamorphosis [M.Sc. thesis]. Montreal, PQ, Canada: Concordia University, 73 p.
Temple HJ, and Cox NA. (2009a). European Red List of Amphibians. Luxembourg: Office for Official Publications of the European Communities,
Temple HJ, and Cox NA. (2009b). European Red List of Reptiles. Luxembourg: Office for Official Publications of the European Communities,
Thomas P, and Sweatman J. (2008). Interference by atrazine and bisphenol-A with progestin binding to the ovarian progestin membrane receptor and induction of oocyte maturation in Atlantic croaker. Mar Environ Res, 66, 1–2.
Tierney KB, Singh CR, Ross PS, and Kennedy CJ. (2007). Relating olfactory neurotoxicity to altered olfactory-mediated behaviors in rainbow trout exposed to three currently-used pesticides. Aquat. Toxicol, 81, 55–64.
Tierney KB, Sampson JL, Ross PS, Sekela MA, and Kennedy CJ. (2008). Salmon olfaction is impaired by an environmentally realistic pesticide mixture. Environ Sci Technol, 42, 4996–5001.
Tillitt DE, Papoulias DM, Whyte JJ, and Richter CA. (2010). Atrazine reduces reproduction in fathead minnow (*Pimephales promelas*). Aquat. Toxicol, 99, 149–59.
Trute M, Gallis B, Doneanu C, Shaffer S, Goodlett D, and Gallagher E. (2007). Characterization of hepatic glutathione S-transferases in coho salmon (*Oncorhynchus kisutch*). Aquat Toxicol, 81, 126–36.
University of Massachusetts. (2011). 2011–2012 New England Guide to Weed Control in Turfgrass. Amherst, MA, USA: Cooperative Extension System. University of Massachusetts. 8 p. www.extension.umass.edu/turf/sites/turf/files/fact./weed_guide_web_11.pdf
[USEPA] Environmental Fate and Effects Division, Office of Pesticide Programs, USEPA. 2001. GENEEC - (GEN)eric (E)stimated (E)nvironmental ©oncentration model [computer program]. Version 2 Washington, DC, USA: USEPA. http://www.epa.gov/oppefed1/models/water/geneec2_users_manual.htm
USEPA. (2002). A Short-Term Method for Assessing the Reproductive Toxicity of Endocrine Disrupting Chemicals Using the Fathead

Minnow (*Pimephales promelas*). Duluth, MN, USA: U.S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division. No. EPA/600/R-01/067. 170 p. http://nepis.epa.gov/Exe/ZyPURL.cgi?Dockey = 30002HB8.txt

USEPA. (2003). A Set of Scientific Issues Being Considered by the Environmental Protection Agency Regarding: Potential Developmental Effects of Atrazine on Amphibians. Washington, DC, USA: United States Environmental Protection Agency. Report of the FIFRA Scientific Advisory Panel Meeting, June 17–20, 2003, No. SAP Report No. 2003–01. 34 p. www.epa.gov/scipoly/sap/index.htm

USEPA. (2005). Draft Final Report on Multi-Chemical Evaluation of the Short-Term Reproduction Assay with the Fathead Minnow. Washington, DC, USA: United States Environmental Protection Agency. No. 68-W-01-023. 165 p. http://www.epa.gov/scipoly/oscpendo/docs/edmvac/draft_fish_repro_multichemical.pdf

USEPA. (2006). Decision Documents for Atrazine. Washington, DC: United States Environmental Protection Agency, Office of Prevention, Pesticides, and Toxic Substances. 323 p. www.epa.gov/oppsrrd1/REDs/atrazine_combined_docs.pdf

USEPA. (2007a). FIFRA Scientific Advisory Panel Meeting, October 9–11, A Set of Scientific Issues Being Considered by the Environmental Protection Agency Regarding: The Potential for Atrazine to Affect Amphibian Gonadal Development. Arlington, VA, USA: United States Environmental Protection Agency. No. FIFRA SAP Minutes No 2007–08. 32 p. http://www.epa.gov/scipoly/SAP/meetings/2007/october/finalminutes.pdf

USEPA. (2007b). White Paper on the Potential for Atrazine to Affect Amphibian Gonadal Development. Washington, DC, USA: United States Environmental Protection Agency. Office of Prevention, Pesticides and Toxic Substances, Office of Pesticide Programs, Environmental Fate and Effects Division. 321 p. http://www.epa.gov/scipoly/sap/meetings/2007/october/2007_amphibian_white_paper.pdf

USEPA. (2011). Evaluation Guidelines for Ecological Toxicity Data in the Open Literature. Procedures for Screening,Viewing, and Using Published Open Literature Toxicity Data in Ecological Risk Assessments. Washington DC: U.S. Environmental Protection Agency, Office of Pesticide Programs. 74 p.

USEPA. (2012a). Guidance for Considering and Using Open Literature Toxicity Studies to Support Human Health Risk Assessment. Washington DC: U.S. Environmental Protection Agency, Office of Pesticide Programs. 16 p.

USEPA. (2012b). Meeting of the FIFRA Scientific Advisory Panel on the Problem Formulation for the Environmental Fate and Ecological Risk Assessment for Atrazine. Washington, DC: United States Environmental Protection Agency, Office of Pesticide Programs. 173 p. http://www.regulations.gov/#!docketDetail;dct = F R % 2 5 2 B P R % 2 5 2 B N % 2 5 2 B O % 2 5 2 B S R ; r p p = 25;po = 0;D = EPA-HQ-OPP-2012-0230

USEPA. (2012c). Scientific Advisory Panel Meeting. A Set of Scientific Issues Being Considered by the Environmental Protection Agency Regarding: Problem Formulation for the Reassessment of Ecological Risks from the Use of Atrazine June 12–14, 2012. Arlington, VA, USA: United States Environmental Protection Agency. No. FIFRA SAP Minutes No 2012–05. 82 p.

USEPA. (2013). State of the Science Evaluation: Nonmonotonic Dose Responses as they Apply to Estrogen, Androgen, and Thyroid Pathways and EPA Testing and Assessment Procedures. Washington, DC: U.S. Environmental Protection Agency, U.S. Environmental Protection Agency & Office of Chemical Safety and Pollution Prevention. No. Draft Report V7. 178 p.

[USGS] United States Geological Survey. (2013). NAWQA Database. USGS website http://cida.usgs.gov/nawqa_public/apex/f?p = 136:1:0, Accessed July 2 2013.

Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs J, David R, et al. (2012). Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocrine Rev, 33, 378–455.

Vredenburg VT. (2004). Reversing introduced species effects: Experimental removal of introduced fish leads to rapid recovery of a declining frog. Proc Nat Acad Sci USA, 101, 7646–50.

Walker E, Hernandez AV, and Kattan MW. (2008). Meta-analysis: Its strengths and limitations. Cleve Clin J Med, 75, 431–9.

Walters AD, Chamberlain K, Ford NB, and Placyk JS, Jr. (2013). Influence of atrazine on the scalation of Marcy's checkered gartersnake, *Thamnophis m. marcianus* (Baird and Girard, 1853). Bull Environ Contam Toxicol, 92, 1–5.

Wan MT, Buday C, Schroeder G, Kuo J, and Pasternak J. (2006). Toxicity to *Daphnia magna*, *Hyalella azteca*, *Oncorhynchus kisutch, Oncorhynchus mykiss, Oncorhynchus tshawytscha,* and *Rana catesbeiana* of atrazine, metolachlor, simazine, and their formulated products. Bull Environ Contam Toxicol, 76, 52–8.

Wang LL, Liu T, Wang C, Zhao FQ, Zhang ZW, Yao HD, et al. (2013). Effects of atrazine and chlorpyrifos on the production of nitric oxide and expression of inducible nitric oxide synthase in the brain of common carp (*Cyprinus carpio* L.). Ecotoxicol Environ Safety, 93, 7–12.

Wang X, Xing H, Li X, and Xu S. (2011). Effects of atrazine and chlorpyrifos on the mRNA levels of IL-1 and IFN-gamma2b in immune organs of common carp. Fish Shellfish Immunol, 31, 126–33

Waring CP, and Moore A. (2004). The effect of atrazine on Atlantic salmon (*Salmo salar*) smolts in fresh water and after sea water transfer. Aquat Toxicol, 66, 93–104.

Weber GJ, Sepulveda MS, Peterson SM, Lewis SS, and Freeman JL. (2013). Transcriptome alterations following developmental atrazine exposure in zebrafish are associated with disruption of neuroendocrine and reproductive system function, cell cycle, and carcinogenesis. Toxicol. Sci, 132, 458–466.

Weed DL. (2005). Weight of evidence: A review of concepts and methods. Risk Anal, 25, 1545–1557.

Wiegand C, Pflugmacher S, Giese M, Frank H, and Steinberg C. (2000). Uptake, toxicity, and effects on detoxication enzymes of atrazine and trifluoroacetate in embryos of zebrafish. Ecotoxicol Environ Safety, 45, 122–31.

Wiegand C, Krause E, Steinberg C, and Pflugmacher S. (2001). Toxicokinetics of atrazine in embryos of the zebrafish (*Danio rerio*). Ecotoxicol Environ Safety, 49, 199–205.

Wieser CJ, and Gross TS. (2002). Determination of Potential Effects of 20 Day Exposure of Atrazine on Endocrine Function in Adult Largemouth Bass (*Micropterus salmoides*). Gainesville, FL: University of Florida, Wildlife Reproductive Toxicology Laboratory. No. Number Wildlife-NOVA98.02e. 83 p.

Williams BK, and Semlitsch RD. (2010). Larval responses of three midwestern anurans to chronic, low-dose exposures of four herbicides. Arch Environ Chem Toxicol, 58, 819–27.

Willingham EJ. (2005). The effects of atrazine and temperature on turtle hatchling size and sex ratio. Front Ecol Environ, 3, 309–13.

Witschi E. (1929). Studies on sex differentiation and sex determination in amphibians. III. Rudimentary hermaphrodites and Y chromosome in Rana temporaria. J Exp Zool, 54, 157–222.

Witschi E. (1930). Studies on sex differentiation and sex determination in amphibians. J Exp Zool, 56, 149–65.

Witschi E. (1942). Temperature factors in the development and the evolution of sex. Biol Symposia, 6, 51–70.

Wolf JC. (2012). Study to Satisfy the EPA Test Order # - EDSP-080803–1,2,3,4 Guideline Study 890.1100 Amphibian Metamorphosis Assay (AMA) Data Requirement for Atrazine Using Existing Frog Tissues Available from Kloas et al. Studies. Greensboro, NC: Syngenta Crop Protection, LLC. No. EPL Project No. 140-121. 34 p.

Woudneh MB, Ou Z, Sekela M, Tuominen T, and Gledhill M. (2009a). Pesticide multiresidues in waters of the Lower Fraser Valley, British Columbia, Canada. Part II. Groundwater. J Environ Qual, 38, 948–54.

Woudneh MB, Ou Z, Sekela M, Tuominen T, and Gledhill M. (2009b). Pesticide multiresidues in waters of the Lower Fraser Valley, British Columbia, Canada. Part I. Surface water. J Environ Qual, 38, 940–7.

Xing H, Wang J, Li J, Fan Z, Wang M, and Xu S. (2010a). Effects of atrazine and chlorpyrifos on acetylcholinesterase and carboxylesterase in brain and muscle of common carp. Environ Toxicol Pharmacol, 30, 26–30.

Xing H, Han Y, Li S, Wang J, Wang X, and Xu S. (2010b). Alterations in mRNA expression of acetylcholinesterase in brain and muscle of common carp exposed to atrazine and chlorpyrifos. Ecotoxicol Environ Safety, 73, 1666–70.

Xing H, Li S, Wang Z, Gao X, Xu S, and Wang X. (2012a). Histopathological changes and antioxidant response in brain and kidney of common carp exposed to atrazine and chlorpyrifos. Chemosphere, 88, 377–83.

Xing H, Li S, Wang Z, Gao X, Xu S, and Wang X. (2012b). Oxidative stress response and histopathological changes due to atrazine and chlorpyrifos exposure in common carp. Pestic Biochem Physiol, 103, 74–80.

Xing H, Wang X, Sun G, Gao X, Xu S, and Wang X. (2012c). Effects of atrazine and chlorpyrifos on activity and transcription of glutathione S-transferase in common carp (*Cyprinus carpio* L.). Environ Toxicol Pharmacol, 33, 233–44.

Yang L, Zha J, Zhang X, Li W, Li Z, and Wang Z. (2010a). Alterations in mRNA expression of steroid receptors and heat shock proteins in the liver of rare minnow (*Grobiocypris rarus*) exposed to atrazine and p,p'-DDE. Aquat Toxicol, 98, 381–7.

Yang L, Zha J, Li W, Li Z, and Wang Z. (2010b). Atrazine affects kidney and adrenal hormones (AHs) related genes expressions of rare minnow (*Gobiocypris rarus*). Aquat. Toxicol, 97, 204–11.

Supplementary material available online

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Zaya RM, Amini Z, Whitaker AS, Kohler SL, and Ide CF. (2011a). Atrazine exposure affects growth, body condition and liver health in *Xenopus laevis* tadpoles. Aquat. Toxicol, 104, 243–53.

Zaya RM, Amini Z, Whitaker AS, and Ide CF. (2011b). Exposure to atrazine affects the expression of key genes in metabolic pathways integral to energy homeostasis in *Xenopus laevis* tadpoles. Aquat. Toxicol, 104, 254–62.

Zhu L, Dong X, Xie H, Wang J, Su J, and Yu C. (2010). DNA damage and effects on glutathione-S-transferase activity induced by atrazine exposure in zebrafish (*Danio rerio*). Environ. Toxicol, 26, 480–8.

Zhu LS, Shao B, Song Y, Xie H, Wang J, Wang JH, et al. (2011). DNA damage and effects on antioxidative enzymes in zebra fish (*Danio rerio*) induced by atrazine. Toxicol Mech Methods, 21, 31–6.